Characterization of the PGE_2 Receptor Subtypes and the IP Receptor in the Rat Medullary Thick Ascending Limb

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Thesis submitted for the degree of
Masters of Science from
the Department of Physiology
Faculty of Medicine
University of Ottawa

September 1996

ISBN 0-612-20002-7

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DEDICATION

This thesis is dedicated to what I consider to be the heart beat of the Children's Hospital of Eastern Ontario: the staff, the patients and their families.

DECLARATION

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Acknowledgments

On the eve of submission of this thesis, I write my acknowledgments as I reflect upon the last two years.

Three people whom I would like to first and foremost thank are Dr. Richard Hébert, Dr. Linda Peterson and Ms. Carol Dryburgh. Richard and Carol helped me make one of the most important decisions in my life two years ago. Without their influence, the events of today, nor my established career goals would most likely not be transpiring. Richard and Linda have given me motivation to explore academic medicine and sharpened my critical reasoning to conduct this pursuit.

I would like to thank the staff in the Emergency Department at the Children's Hospital of Eastern Ontario. Their continued support and well wishes have often been a much-needed energy reserve. Their's will be the standard by which I will measure professionalism and teamwork.

I would also like to thank my friends, both past and current. I will cherish the many Friday evenings spent with Dave, Christine, Mike, Simon and Pete from the Cancer Research Group. There will be no shortage of memories of times spent at the Heart and Crown with friends from the Biology Department including Heather, Gary, Wendy and Tim. Mike, Martin, Curt and Sandrine from the Chemistry Department have always been there for me and I'm sure will continue to be a source of Canada Day entertainment. I would also like to mention my appreciation for my close friends in the Physiology Department including Harman, Laura, Agnes, Chris, Mark, Joe and Jimmy.

In closing, I want to thank my parents Sarsfield and Sheila O'Connor. Their endless support and encouragement has taught me never to give up. What I have to say on their behalf can best be told in the words of Irish poet Seamus Heaney:

"...Believe that a further shore is reachable from here
Believe in miracles, and cures, and healing wells..."

T.M.O'C.

ABSTRACT

Characterization of the PGE_2 Receptor Subtypes and PGI_2 Receptor in the Rat Medullary Thick Ascending Limb

PGE_2 attenuates AVP-dependent solute transport in the medullary thick ascending limb of Henle (mTAL) and like the PGI_2 I-prostanoid (IP) receptor inhibits AVP-dependent water flow in the collecting duct. PGI_2 may play a role similar to PGE_2 in the mTAL. We therefore further characterize E-prostanoid (EP) receptor subtypes and the IP receptor in a freshly isolated suspension of rat mTAL. Using 10 nM PGE_2, we confirmed previous work showing a Gi coupled EP_3 receptor subtype in rat mTAL that attenuates AVP-dependent cAMP by 70.7%. Pertussis toxin reversed this inhibitory response by 73.2%. In the absence of AVP, micromolar concentrations of PGE_2 and the EP_2 receptor subtype specific agonist butaprost produced significant elevations in cAMP levels, indicating the existence of the G_s-coupled EP_2 receptor subtype. This response was insensitive to the EP_4 receptor subtype antagonist AH-23848B. Using the PGI_2 analogs iloprost (ILP) and cicaprost (CCP), we have demonstrated the presence of a Gi-coupled PGI_2 receptor. AVP-dependent cAMP was reduced 80% and 68.9% by 0.1uM ILP and 1 uM CCP respectively. Pertussis toxin reversed these inhibitory actions by 73%. Neither the EP_3 receptor subtype nor IP receptor inhibitory responses on AVP-dependent cAMP were sensitive to the EP_1 receptor subtype specific antagonist AH-6809 and the protein kinase C (PKC) inhibitors bisindolylmaleimide I and calphostin C. By associating in situ hybridization for receptor mRNA with TAL-specific Tamm-Horsfall glycoprotein immunostaining on serial sections from rat kidney, we have clearly shown EP_3 receptor subtype and IP receptor expression in the rat mTAL. These results suggest that a Gi-coupled IP receptor and the EP_3 receptor subtype play a role in attenuating the AVP-dependent cAMP in the rat mTAL.

Table of Contents

Section 1: Introduction

Section 2: Materials and Methods

Section 3: Results

Section 4: Discussion

Section 5: References

List of Figures

List of Abbreviations

°C	degrees Celsius
AC	adenylate cyclase
AH-23848B	EP_4 receptor subtype antagonist
AH-6809	EP_1 receptor subtype specific antagonist
ANOVA	analysis of variance
ATP	adenosine triphosphate
AVP	arginine vasopressin
AVP-Lp	arginine vasopressin-dependent hydraulic conductivity
BIS.1	PKC inhibitor
BSA	bovine serum albumin
BUT	butaprost
c-PGI_2	carba-prostacyclin
cAMP	cyclic adenosine monophosphate
cc	cubic centimeter
CCD, MCD	cortical, medullary collecting duct
CCP	cicaprost
cDNA, DNA	complimentary deoxyribonucleic acid
CHO	Chinese hamster ovary cells
cox	cyclo-oxygenase
CPM	counts per minute
DAB	diamino benzidine
DAG	diacyl glycerol
dDAVP	1-desamino-8-D-arginine vasopressin
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
DL	dilution ratio
DTT	dithiothreitol
EGF	epidermal growth factor
EP_1, EP_2, EP_3, EP_4	E-prostanoid receptor subtypes
EtOH	ethanol
g	gravitational force
GC	guanine-cytosine
Gi	inhibitory guanine nucleotide regulatory protein
Gs	stimulatory guanine nucleotide regulatory protein
H-7	PKC inhibitor
HBSS	Hank's balanced salt solution
IgG	immunoglobulin G
IL-1	interleukin-1
ILP	iloprost
IP	I-prostanoid receptor
IP_3	1,4,5-triphosphate
MeOH	methanol
mOsm	milliosmoles

mRNA	messenger ribonucleic acid
mTAL, cTAL	medullary, cortical thick ascending limb
N	normality
NCBI	national center for biotechnology information
ng, ug, mg, kg	nano, micro, milli, kilogram
nm,um,cm	nano, micro, centimeter
NTB2	nuclear tract emulsion B2
OD	optical density
ODU	optical density unit
PA, LPA	phosphatidic acid, lysophosphatidic acid
PBS	phosphate buffered saline
PC, I	phosphatidyl choline, inositol
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGHS	prostaglandin H synthase
PGI_2	prostaglandin I_2, prostacyclin
PIP_2	inositol-4,5-bisphosphate
PKA	protein kinase A
PLC, D	phospholipase C, D
PMA	phorbol myristate acetate
PT	proximal tubule
PTH	parathyroid hormone
PTX	pertussis toxin
Ro20-1724	phosphodiesterase inhibitor
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SSC	saline sodium citrate
TdT	terminal deoxynucleotidyl transferase
TEA	triethanolamine
TH	Tamm-Horsfall glycoprotein
TNF	tumor necrosis factor
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
ul, ml	micro, milliliter
uM,M	micromolar, molar
V_1, V_2	vasopressin receptor subtypes

Section 1: Introduction

1.00 Introduction

This project focuses on characterizing prostaglandin receptors and receptor subtypes, particularly PGE_2 and PGI_2, in the rat medullary thick ascending limb (mTAL). E-prostanoid (EP) receptor subtypes have been well characterized in various tissues including the kidney (Dunn and Hood 1977, Ferris 1983, Thieruch et al. 1993, Breyer et al. 1996). I-prostanoid receptors are likewise referred to as IP receptors. Prostanoid receptors and their receptor subtypes exert their different biological effects through various cellular signaling mechanisms. These receptors are typically coupled through G regulatory proteins to membrane bound enzymes which generate second messenger molecules that begin a cellular signaling cascade. When different receptor subtypes for a given prostanoid exist on the same tissue, cell signaling cross talk can occur whereby one signaling pathway can modulate another (Ando et al 1988, Tietelbaum 1990, Kozawa et al. 1992). This may represent a form of feedback inhibition regulating prostanoid-mediated biological responses. PGE_2 is the most abundant renal prostanoid, and its inhibitory effect on AVP-dependent solute reabsorption in the mTAL has been well characterized (Erikson et al. 1987, Bonvalet et al. 1987, Ando et al. 1988). PGI_2 may play a similar, though less significant, role in the mTAL. These mechanisms of prostanoid action in the kidney are important in renal disease because elevated PGE_2 levels observed during chronic hypercalcemia inhibit AVP-dependent solute reabsorption (Berl 1987, Peterson 1990, Peterson et al. 1993). This may be an important component in the pathophysiology underlying the urinary concentrating defect observed in nephrogenic diabetes insipidus (Rose and Renke 1994).

1.01 The Kidney

The kidney is composed of an outer cortex and inner medulla. The latter region is divided into an outer stripe, an inner stripe, and the renal papilla from which the renal artery enters, and renal vein and ureter exit. Regions of the nephron, the functional unit of the kidney, are strategically located within portions of the kidney with unique osmotic characteristics to fulfill functional objectives. As well, the functional heterogeneity of the nephron also depends on the unique epithelial morphology of a given tubule segment within its respective osmotic environment. The morphology of the apical and basolateral membranes of different tubular segments changes with regards to the solute channels, transporters and intercellular junctions present (Koeppen and Stanton 1992).

The glomerulus is located in the well vascularized cortical region and is the site of plasma filtration. The osmolarity of the glomerular filtrate, 300 mOsm, is equal to that of plasma. Isosmotic solute and water reabsorption occurs in the proximal tubule, also located in the cortex. Distal to the proximal tubule, the descending limb of Henle moves down into the outer and inner medullary regions. At the cortical-medullary boundary the interstitial osmolarity is still equivalent to that of plasma; however, the osmolarity progressively increases further down into the medulla to reach a value of 1200 mOsm deep within the inner medulla. It is through this environment that the Loop of Henle must descend and ascend. This coupled with the different permeability characteristics of the descending and ascending limbs, determines the direction of fluid and solute movement. The descending thin limb is passively permeable to water which is drawn by interstitial osmotic forces from the tubular lumen to the medullary interstitium. Luminal osmolarity increases

as it continually equilibrates with the increasing osmolarity of the adjacent interstitium. The ascending limb is composed of thin and thick regions. Intercellular tight junctions makes this region impermeable to water. The thin portion is passively permeable to NaCl which moves out of the lumen down its concentration gradient as the limb ascends into regions of decreasing interstitial osmolarity; the thick ascending limb, using active transport pumps, moves solute from the tubule lumen into the medullary interstitium against the NaCl concentration gradient. It is important to note that through active transport, the thick ascending limb develops and maintains the high medullary interstitial osmolarity which would otherwise be dissipated by vasa recta blood flow. This high medullary interstitial osmolarity is important because it provides the osmotic driving force necessary to facilitate water reabsorption from the collecting duct (CD). The CD is the terminal region of the nephron and is the last site for modification of tubular fluid before entering the ureters (Koeppen and Stanton 1992, Rose 1994).

1.02 The Thick Ascending Limb

The thick ascending limb (TAL) is divided into medullary (mTAL) and cortical (cTAL) portions. Both serve to increase the medullary interstitial osmotic gradient and dilute the tubular fluid by active transport of luminal salts to the interstitium. Furosemide sensitive $Na^+/K^+/2Cl^-$ symporters are located in the apical membrane of TAL. K^+ is carried down the Na^+ and Cl^- concentration gradients from lumen to cytoplasm. This apical symporter is indirectly driven by the active transport of the ouabain sensitive Na^+/K^+-ATPase ion pump on the basolateral membrane which extrudes three Na^+ in exchange for two K^+, thereby maintaining a low intracellular Na^+

concentration. K^+ channels on the apical membrane allow this ion to move down its concentration gradient into the tubular lumen. This, together with Cl^- movement out of the basolateral membrane and the net loss of one positive charge (= one negative charge gained) generated by one cycle of the basolateral Na^+/K^+-ATPase, creates a transepithelial lumen positive voltage difference. This voltage is proportional to the activity of the $Na^+/K^+/2Cl^-$ symporter. Although the tight junctions which exist between adjacent cells make the mTAL impermeable to water, they do allow the movement of Na^+, K^+, Ca^{2+}, and Mg^{2+} driven by this lumen positive potential difference. This is called paracellular ion transport and accounts for 50 % of the salts reabsorbed. The mTAL reabsorbs 20 % of the filtered load of Na^+, Cl^-, HCO_3^-, K^+ and Ca^{2+}, and 80 % of the filtered load of Mg^{2+} (Burg 1982, Molony et al. 1989, Koeppen and Stanton 1992).

Although the PT and CD are landmark regions of the nephron for acid-base regulation, the mTAL also contributes to metabolic acid-base balance (Burg 1982, Good 1989, Koeppen and Stanton 1992, Rose 1994). This occurs through HCO_3^- and NH_4^+ reabsorption. The cytoplasm of mTAL epithelia contains carbonic anhydrase (CA) which catalyses the conversion of H_2O and CO_2 to H_2CO_3. $H_2CO_3^-$ dissociates to H^+ and HCO_3^-. H^+ are pumped against their concentration gradient into the lumen by apical membrane, amiloride sensitive Na^+-K^+ antiporters, while the HCO_3^- are reabsorbed across the basolateral membrane, a process attenuated by arginine vasopressin (AVP). NH_4^+, a product of glutamine metabolism, is secreted by the proximal tubule to replenish the loss of filtered HCO_3^- used to titrate non-volatile acids in the tubule lumen. The NH_4^+ is reabsorbed in place of K^+ on the Na^+-K^+-$2Cl^-$ symporter in the TAL apical membrane. NH_4^+ crosses the basolateral membrane and accumulates in the medullary interstitium, where it dissociates into NH_3 and H^+. NH_3 is secreted into the lumen of the CD where it becomes

protonated to reform cationic ammonia in the tubular lumen. Through a process called ammonia trapping, the NH_4^+ cannot diffuse freely back across the apical membrane of the collecting duct. The net result is one H^+ excreted and one HCO_3^- reabsorbed. NH_4^+ reabsorption is sensitive to filtered K^+ as higher concentrations of this ion will compete with NH_4^+ for the same binding site on the Na^+-K^+-$2Cl^-$ symporter. Both forms of mTAL acid-base regulation are sensitive to chronic metabolic acidosis.

The TAL, both medullary and cortical, is composed of only one cell type. Although there is no histological heterogeneity, there is variation in this cell type organized around structural-functional relationships from medullary to cortical regions (Allen and Tisher 1976, Madsen and Tisher 1986). As the TAL moves upward through this region, the height of the cells gradually decreases from 7-8 μm in the inner medullary stripe to 5 μm in the cortex. In this direction, the surface area of the apical membrane progressively increases while that of the basolateral membrane progressively decreases. These patterns of change in cell size and surface area correlate with the finding that Na^+/K^+-ATPase activity is greater at the medullary inner stripe than at the cortical-medullary boundary; the basolateral membrane of the ascending TAL provides less surface area for Na^+/K^+-ATPase pumps. This is in agreement with functional evidence from NaCl transport studies which show a greater rate of NaCl transport in the inner medullary portion of the TAL. Despite this however, the cTAL is capable of generating a lower tubular NaCl concentration. Other variations in TAL from medulla to cortex include shortened mitochondria, increased number of microvilli, and greater interdigitation between adjacent cells.

Immunocytochemical studies involving the TAL use antibodies to the Tamm-Horsfall (TH) glycoprotein (Breyer et al. 1993). TH is a 639 amino acid urinary glycoprotein synthesized by the

the TAL. It is located on both apical and basolateral membranes and throughout the cytoplasm from the inner stripe of the medulla to the early distal convoluted tubule past the macula densa (Hoyer et al. 1979). An immunoregulatory role has been proposed for TH glycoprotein (Hession et al. 1987). The glycoprotein can bind interleukin-1 (IL-1) and tumor necrosis factor (TNF) and may be a ligand for these circulating lymphokines on the basolateral membrane. Once bound, the TH-lymphokine complex may become internalized and secreted into the tubular lumen across the apical membrane. This complex can then be excreted in the urine. TH isolated from the urine of pregnant women was shown to be more immunosupressive compared to the same product from non-pregnant women. Also, the carbohydrate portion of the protein is necessary for biological activity. The glycosylated protein will bind IL-1 and TNF and can supress antigen-specific T-cell proliferation in vitro (Hession et al. 1987).

1.03 Hormonal Regulation

Probably the most functionally important regulatory hormone of the TAL is arginine-vasopressin (AVP). In vitro and in vivo microperfusion studies have shown that physiological concentrations of AVP (1-10pM) are responsible for regulating the activity of the $Na^+/K^+/2Cl^-$ symporter by increasing the number of active pumps (Burg 1982). AVP effectively increases the medullary interstitial osmotic gradient underlying the urinary concentrating mechanism by increasing the number of active transporters, (Jamison and Maffly 1976). Another important function of AVP in the kidneys is to stimulate the insertion of water channels into the apical membrane of the CD (Deen et al. 1994, Sabolic and Brown 1995). These pumps provide the

gateway for water diffusion out of the tubule lumen, driven by the augmented medullary interstitial osmolarity. While the functional effect of AVP to reduce urinary water loss is important, there are species differences of which to make note. The ability of AVP to stimulate TAL solute reabsorption occurs in rabbit, rat, and mouse, but not in human and dog kidneys (Morel 1981, Morel et al. 1987). This activity of AVP seems to correlate well with species that produce a more concentrated urine. It is important to note that in species where this AVP effect is observed, TAL luminal fluid is diluted through salt removal in the absence of water reabsorption.

The mechanism of AVP action on TAL is through the cAMP second messenger system (de Rouffignac et al. 1991). Acting on the V_2 receptor subtype coupled to a G stimulatory (Gs) protein, AVP stimulates adenylate cyclase (AC) (Morel et al. 1987). This membrane-bound enzyme is responsible for the conversion of ATP to cAMP. Cyclic AMP can then stimulate cAMP-dependent protein kinases such as protein kinase A (PKA), resulting in the second messenger cascade leading to a physiological effect. Further evidence indicating cAMP as the second messenger molecule shows that cAMP analogs also stimulate NaCl reabsorption in the TAL and the NaCl reabsorptive responses to cAMP and AVP are not additive. Evidence in support of the V_2 receptor subtype shows that dDAVP, a V_2 receptor subtype specific analog, is equipotent to AVP in stimulating NaCl reabsorption.

Adenylate cyclase (AC) activity can also be stimulated by other peptide hormones. AVP and glucagon can stimulate mTAL AC, while parathyroid hormone (PTH), calcitonin and glucagon can stimulate AC activity in the cTAL of the rat (Amiel et al. 1987). At maximal concentrations these hormones do not exert additive effects on AC stimulation, nor cAMP accumulation, suggesting these hormones act on the same coupling mechanisms. In vitro

micropuncture studies show all four peptide hormones to produce similar effects in TAL function (deRouffiganc et al. 1987). Brattleboro diabetes insipidus rats with thyroparathyroidectomy and somatostatin treatment were used in these studies to eliminate endogenous sources of these hormones. Further in vitro microperfusion studies confirmed these results. PTH, glucagon and calcitonin may facilitate a basal level of NaCl reabsorption that may be necessary to maintain medullary interstitial osmolarity given that circulating AVP levels can fluctuate significantly within minutes. Consider AVP acting alone: after acute water load AVP levels might drop to a point where a basal level of medullary interstitial osmolarity can no longer be maintained. This osmolarity can drop within hours yet require several days to accumulate to normal physiological levels. This is clearly too long, so some combination of the other peptide hormones may play a role in maintenance of a tonic level of medullary interstitial osmolarity, which can be augmented relatively quickly through increased circulating levels of AVP (Kurokawa et al. 1992).

The TAL response to AVP is subject to desensitization following repeated and prolonged exposure. This desensitization is believed to occur at the receptor level because AC is still responsive to calcitonin and glucagon even when the enzyme response to AVP has become desensitized (Deblineau et al. 1989). The mechanism of action for desensitization, although not completely understood, may be similar to that of the adrenoreceptors. When activated, the second messenger response from adrenoreceptor-agonist complex phosphorylates tyrosine residues on the receptor, resulting in internalization of the receptor-agonist complex (Kurokawa 1992).

PGE_2 plays an inhibitory role on the dual activity of AVP in the kidney (Smith et al. 1989). PGE_2 reduces both AVP-dependent water flow in the CD and AVP-dependent NaCl reabsorption in the mTAL. This inhibitory action of PGE_2 is believed to occur at a post receptor site because

the same inhibitory action is observed using PTH, calcitonin and glucagon (Torikai and Kurokawa, 1983). The effect of the peptide hormones is reinstated with the addition of cAMP, suggesting that PGE_2 acts at a pre-cAMP site (Culpepper and Andreoli 1983). This combined with the evidence that PGE_2 inhibition of AVP action can be partially reversed with pertussis toxin, implies that the PGE_2 mechanism of action involves an inhibitory G-regulatory protein (Gi) that attenuates the activity of AC.

1.04 Prostanoid Synthesis and Actions

The prostaglandins are a family of biologically active lipid compounds and form the end product of membrane lipid metabolism. Hormone-induced signals cause the release of esterified arachidonate from eicosatetraenoic acid. Cyclooxygenase (cox), also known as prostaglandin H Synthase (PGHS) converts the arachidonic acid to PGH_2 in a two step reaction producing PGG_2 first which has its 15-hydroperoxy group reduced. PGH_2 is then oxygenated by isomerases and reductases to the various prostanoids including PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2 (Smith et al. 1991, Smith 1992). Prostanoids leave the cell by facilitated diffusion and act in a autocrine or paracrine fashion. Chemical instability limits the plasma half-life; therefore, the prostanoids act as paracrine and autocrine agents.

Prostanoids exhibit a diverse range of biological activities in numerous tissues throughout the body (Smith 1989, Smith 1992). While at first, new drug therapies using prostaglandins looked promising, the realization of equally potent side effects impeded progress. Prostanoids have a wide range of biological effects. PGE_2 mediates both contraction and relaxation of smooth

muscle, and both attenuates and increases neurotransmitter release. PGE_2 also inhibits lipolysis, gastric acid secretion, renal water excretion, inflammatory mediator release and is involved in immunoregulation. The different and sometimes opposing biological activities for a given prostanoid are mediated by receptor subtypes coupled to alternative cellular signaling mechanisms.

The kidney is an organ well endowed with prostanoid activity. PGE_2 is the most abundant renal prostanoid with intrarenal concentrations reaching nanomolar values, while circulating levels are in the picomolar range. In the kidney, PGE_2 is about twenty times more abundant than 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2 and greater than one hundred times more abundant than TxB_2 (Bonvalet et al. 1987). It is difficult to assess tissue concentrations of PGI_2 directly because this prostanoid is chemically unstable at physiological pH (Coleman et al. 1994). All renal prostanoids have approximately the same segmental distribution along the nephron. The main sites of synthesis for renal prostanoids are the medullary collecting duct (CD) followed by the CCD and the thin descending limb of Henle's loop (Smith et al. 1991, Smith 1992). Renal prostanoid synthesis is initiated by enzymatic conversion of membrane lipid to arachidonic acid through the actions of hormones including angiotensin, bradykinin and AVP (Smith et al. 1991, Smith 1992). PGE_2 release mediated by AVP may form a negative feedback loop inhibiting AVP-dependent solute and water reabsorption (Walker et al. 1978). The main biological activities of renal prostaglandins involve regulation of renal hemodynamics and solute and water transport (Smith 1992).

1.05 Prostanoid Receptors and Receptor Subtypes

Prostanoids bind to specific receptors; in the case where one prostanoid produces multiple or opposing biological activities, it is possible that receptor subtypes specific to that prostanoid exist. Four "E" prostanoid or EP receptor subtypes exist: EP_1, EP_2, EP_3 and EP_4 (Coleman et al. 1994). The PGI_2 receptor is are referred to as the I-prostanoid or IP receptor. Prostanoid receptors are coupled to their intracellular effectors by guanine nucleotide regulatory binding proteins (G-proteins). The secondary and tertiary structure of the prostanoid receptor family consists of seven α-helical, hydrophobic membrane spanning domains (Henderson et al. 1990). The receptors bind the third intracellular loop and the proximal region of the C-terminal tail of G-proteins (O'Dowd et al. 1989). The G-proteins couple to one of three intracellular effectors (Breyer et al. 1995, Breyer et al. 1996). Gq proteins couple to PLC, which when activated hydrolizes the plasma membrane phospholipid phosphatidyl inositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG can also be liberated from PLC hydrolysis of membrane phosphatidylcholine (PC). The DAG remains in the plasma membrane and activates protein kinase C (PKC) by increasing its affinity for Ca^{2+}. The activation of PKC then phosphorylates substrate proteins that cascade to their physiological effect within the cell. The water-soluble IP_3 moves through the cytoplasm stimulating the release of Ca^{2+} from intracellular stores, which form Ca^{2+}-calmodulin complexes that associate with calmodulin-dependent enzymes that include various kinases and phosphodiesterases, which ultimately cascade

to their physiological role. It is important to note that, when stimulated by one of its receptor-ligand complexes, the Gq protein can couple to and activate phospholipase D (PLD) as well a PLC. PLD hydrolizes PC and phosphatidyl inositol (PI) to phosphatidic acid (PA), which can be further metabolized to DAG and lysophosphatidic acid (LPA). The Gs-protein couples the receptor to and activates AC, increasing cAMP. The Gi-protein likewise couples the receptor to AC, but inhibits cAMP production.

Prostanoid receptors and receptor subtypes can be examined using a number of techniques including second messenger studies and molecular biology techniques (Coleman et al.1994). Second messenger studies require the use of tools such as prostanoid analogs, agonists and antagonists specific to particular receptor subtypes. Using these tools, the investigator can study the cell signaling responses characteristic to receptor subtypes by assaying for changes in levels of second messenger molecules. Molecular biology techniques allow the investigator to study temporal expression of receptor subtypes in different tissues under different biological conditions. Once a receptor subtype has been cloned and its cDNA sequence reported, nucleic acid probes can be designed to study not only which tissues express the gene for a receptor subtype, but using high resolution emulsion autoradiography the cells within a given tissue that express the gene.

1.06 EP Receptor Subtypes

Various agonists and antagonists exist which are specific to the EP receptor subtypes. Also, all EP receptor subtypes known to date have been cloned and their cDNA sequences have been reported. Both second messenger studies using these agonists and antagonists and in situ

hybridization have been done to provide information about the intrarenal localization of EP receptor subtypes.

The EP_1 receptor subtype is Gq protein coupled to PLC which hydrolyzes PIP_2. AH-6809 is a specific antagonist for the EP_1 receptor subtype (Coleman and Kennedy 1985). Although more potent as an EP_3 agonist, sulprostone was first discovered to have EP_1 potency (Bunce et al. 1990). PGE_2 stimulates an increase in intracellular Ca^{2+} in rabbit cortical collecting duct (CCD) that is independent of extracellular Ca^{2+} and insensitive to pertussis toxin (Breyer et al. 1992). However, this response could be inhibited by 50% using AH-6809 and is believed to be IP_3-independent since no elevations of IP_3 were observed. It was shown in rabbit CCD that PGE_2 attenuates AVP-dependent water flow through Ca^{2+} signaling and a Gi protein coupled mechanism, implicating the EP_1 and EP_3 receptor subtypes respectively (Hébert et al. 1993, Hébert et al. 1995). Arachidonic acid, the PGE_2 precursor, has been shown to increase intracellular Ca^{2+} in the rat mTAL (Firsov et al. 1994). This suggests that the EP_1 receptor subtype may be present in rat mTAL. In situ hybridization has localized expression of EP_1 receptor subtype mRNA to the CCD in mouse and human kidney (Sugimoto et al. 1994, Fowler et al. 1995, Breyer et al. 1996).

Both the EP_2 and EP_4 receptor subtypes are coupled by Gs-proteins to the activation of AC (Henderson et al. 1990, Breyer et al. 1996). While PGE_2 stimulates both receptor subtypes, butaprost is a specific agonist for the EP_2 receptor subtype and AH-23848B is an antagonist for the EP_4 receptor subtype (Gardiner, 1986 and Coleman et al. 1994). Both butaprost and AH-23848B have weak potency for the EP_2 and EP_4 receptor subtypes specifically. Butaprost is ten-fold less potent than PGE_2. Second messenger studies show PGE_2 to increase cAMP in the rabbit

CCD (Sonnenberg and Smith 1988, Hébert et al. 1993); stimulation of the EP_2 receptor subtype may increase water reabsorption in the collecting duct (Schafer and Troutman 1990, Hébert et al. 1993, Breyer et al. 1996). Because PGE_2 has also been shown to increase Ca^{2+} as well as cAMP in CCD cells, the EP_1 and EP_3 selective agonist sulprostone was tested and was found unable to repeat this cAMP response. There is evidence that PGE_2 in the absence of AVP increases cAMP in the mTAL of rat and rabbit (Torikai and Kurokawa 1983, Nakao et al. 1989). PGE_2 also increases glomerular cAMP levels possibly through the EP_4 receptor subtype (Chaudhari et al. 1990, Breyer et al. 1996), which may be responsible for the vasodilatory effects of PGE_2 observed in the glomerular microvasculature (Edwards 1985). In situ hybridization studies have localized both EP_2 and EP_4 receptor subtype mRNA to the glomeruli of human and mouse kidneys (Sugimoto et al. 1994, Breyer et al. 1996).

The EP_3 receptor subtype can be coupled to different cellular signaling mechanisms in various tissues. EP_3 receptor subtypes coupled to different G-proteins are termed EP_3 receptor subtype isoforms (Breyer et al. 1995, Breyer et al. 1996). Their coupling properties are determined by C-terminal modifications resulting from alternative splicing of mRNA (Namba et al. 1993, Adam et al. 1994, Regan et al. 1994). However, in most physiological systems including the renal medulla, the EP_3 receptor subtype is coupled to the Gi-protein. The PGE_2 concentrations required to observe other types of G-protein coupling to the EP_3 receptor subtype are well above physiological range (Breyer et al. 1996). PGE_2 inhibition of AVP-dependent water reabsorption in the rabbit CCD is at least partially due to the coupling action of AC to a Gi protein because PT reverses this effect (Sonnenburg et al. 1990). Commonly used EP_3 agonists include sulprostone, enprostil and misoprostol. Sulprostone is not only EP_3 specific, but can activate the

EP_1 receptor subtype as well. However, enprostill and misoprostol are highly specific and have at least a ten-fold greater potency than PGE_2 for the EP_3 receptor subtype (Coleman et al. 1994). PGE_2 and sulprostone inhibit AVP-dependent cAMP in the isolated rat mTAL (Nakao et al. 1989, Torikai and Kurokawa 1983, 1983, Firsov et al. 1994). This inhibitory effect of PGE_2 in the mTAL has also been shown to be sensitive to PT (Nakao et al. 1989, Firsov et al. 1994). In situ hybridization on mouse, human and rabbit kidney shows EP_3 receptor subtype mRNA to be expressed in the mTAL, MCD and CCD (Breyer et al. 1993, Sugimoto et al. 1994, and Breyer et al. 1996).

1.07 IP Receptor

Similar to the nomenclature for the PGE_2 receptors, PGI_2 receptors are referred to as "I" prostanoid or IP receptors. Currently however, there is debate as to whether or not distinct IP receptor subtypes exist or not. No molecular evidence exists to date, neither homologous molecules nor alternatively spliced variants of a cloned receptor, for distinct IP receptor subtypes. However, one IP receptor may be coupled to different signaling pathways. The cDNA for a prostacyclin receptor has been isolated and cloned from a mouse library using reverse transcriptase polymerase chain reaction (RT-PCR) and hybridization screening (Namba et al. 1994). Following the expression of the receptor in CHO cells, ^{3}H-Iloprost (a PGI_2 agonist) was found to bind with a Kd=4.6nM and stimulate an increase in both cAMP and IP_3. Increases in both cAMP and IP_3 would suggest activation of putative IP_2 and IP_1 receptor subtypes respectively.

Although the molecular evidence presented to date is not in agreement with the existence of IP receptor subtypes for PGI_2, the biochemical evidence at the second messenger level argues otherwise. In cultured rat IMCD cells, PGI_2 at 0.3, 3, and 30 uM has been shown to stimulate increasing levels of cAMP that are independent of AVP and mediated by a receptor distinct from the EP_2 receptor subtype as determined from desensitization studies (Veis et al. 1990). This group did not elaborate on specific receptor subtypes, but a cAMP-generating response implicates a putative IP_2 receptor subtype. In isolated perfused rabbit CCD, the PGI_2 analogs carba-prostacyclin (c-PGI_2) and ILP at 10^{-7}M have been shown to inhibit AVP-dependent hydraulic conductivity (AVP-Lp) and increase intracellular Ca^{2+} (Hébert et al. 1995). This group did conjecture that these two different functional effects are possibly mediated by distinct PGI_2 receptor subtypes. They described the inhibition of AVP-Lp as a putative IP_3 receptor subtype mediated response and the rise in intracellular Ca^{2+} as a putative IP_1 receptor subtype mediated response. The responses from these putative receptor subtypes were differentiated from those of corresponding PGE_2 receptor subtypes through additivity and desensitization experiments.

The PGI_2 analogs used in these studies have their benefits and potential drawbacks. While PGI_2 is the natural agonist of the IP receptor, it has limited chemical stability at physiological pH (Coleman et al. 1994). The c-PGI_2 and ILP analogs bring chemical stability and appreciative duration of action, but c-PGI_2 has limited potency and ILP is also an EP_1 receptor subtype agonist (Sheldrick et al. 1988). A more ideal analog is cicaprost which has a slightly greater potency for the IP receptor than PGI_2 and specificity that is limited or non-existent for the other prostanoid receptor subtypes tested (Sturzebecher et al. 1985).

While the role of PGI_2 is not clearly established in tubular epithelia, it is more definitive with regards to the renal vasculature (Breyer et al. 1996). PGI_2 plays a vasodilator function in the microvasculature of the glomerulus (Edwards 1985). Both PGI_2 and PGE_2 increase cAMP in glomerular microvasculature. Studies showing the additive effects of these responses provide evidence of different prostanoid receptors (Chaudhari et al. 1990). There is limited in situ evidence characterizing the presence of the IP receptor in the mouse renal interlobular arteries and in the smooth muscle cells of the afferent arterioles (Oida et al. 1995).

1.08 Cell Signaling Cross-Talk

The EP receptor subtypes couple through three different cellular signaling mechanisms: EP_1 is coupled to PLC activation and PIP_2 hydrolysis, EP_2/EP_4 to Gs coupling which then activates AC, and EP_3 to Gi coupling which inhibits AC. There is evidence which indicates that there is some interaction between signaling pathways activated by receptor subtypes for PGE_2. This interaction between messenger molecules may provide a form of feedback regulation.

For cell signaling cross-talk to occur, it is necessary for different receptor subtypes coupling to different signal transduction pathways to be present on the same part of the tubule. EP_1, EP_2, and EP_3 receptor subtypes have been characterized in the rabbit CCD. Studies on rat IMCD cells have shown PKC to attenuate cAMP levels (Tietelbaum 1990). These studies used epidermal growth factor (EGF) and DAG as activators of PKC. While a reduction in cAMP was achieved, this PKC effect was abolished with the PKC inhibitor H-7. Similar results were obtained in studies on osteoblast-like cells (Kozawa et al. 1992). In these cells, PGE_2 increased

both cAMP and PKC levels, indicating the presence of EP_2 and EP_1 receptor subtypes respectively. A PKC activating phorbol ester attenuated cAMP levels while the AC activator forskolin had no reciprocal effect on IP_3 formation. H-7 increased cAMP levels in comparison to controls. Both of these studies provide evidence of signaling cross talk, possibly as a means of PGE_2 feedback autoregulation.

While the signaling cross-talk studies mentioned above concerned regulation between EP receptor subtypes, there is also evidence that more than one receptor subtype is involved in the modulating actions of PGE_2 on AVP activity. In rabbit CCD, PGE_2 inhibition of AVP-dependent water flow has been shown to be mediated by EP_1 and EP_3 receptor subtypes (Hébert et al. 1993). Another study found PGE_2 inhibition of AVP-dependent cAMP to have both staurosporine (a PKC inhibitor) and PTX sensitive components in rabbit CCD (Noland et al. 1992). This evidence suggests that both EP_1 and EP_3 receptor subtypes play a role in modulating AVP-dependent cell signaling responses.

Similar signaling cross-talk might occur in the mTAL Different EP receptor subtypes would have to be characterized on this region of the nephron. Already there is cell signaling evidence for the presence of EP_2 and EP_3 receptor subtypes, and in situ hybridization demonstrates the expression of the EP_3 receptor subtype (Breyer et al. 1993, Sugimoto et al. 1994). There is also limited evidence suggesting the presence of an EP_1 receptor subtype where the PGE_2 precursor arachidonic acid was found to increase intracellular Ca^{2+} in rat mTAL.

1.09 Pathophysiology and Clinical Significance

The actions of elevated PGE_2 levels have been implicated in the mechanism underlying the renal concentrating defect observed in chronic hypercalcemia. Chronic hypercalcemia leads to one form of nephrogenic diabetes insipidus that arises from impaired renal response to AVP, resulting in polyurea (Rose and Renke 1994). Because of this disorder, it is important that prostanoid receptor characterization in the mTAL be further understood.

At present, the mechanisms underlying this process are incompletely understood. It has been shown that vitamin D induced hypercalcemic rats have decreased mTAL NaCl reabsorption (Peterson 1990). In both the hypercalcemic model and in cells incubated in high Ca^{2+} media, thick ascending limb AVP-dependent cAMP is impaired (Takaichi and Kurokawa 1986, Berl 1987). It was postulated that the decreased ability of AVP to stimulate sufficient solute reabsorption from the mTAL might compromise medullary hypertonicity. Because this effect is also observed using glucagon and forskolin, the underlying mechanism was believed to occur at a post receptor site.

While the pathophysiology underlying hypercalcemia induced polyurea was initially thought to be prostanoid independent (Levi et al. 1983), further investigation showed otherwise. PGE_2 concentrations in the outer medulla of chronic hypercalcemic rats are nine to ten fold greater compared to controls; in the hypercalcemic rats, chloride reabsorption was 20% less. Despite continuation of the hypercalcemic state, the impaired chloride reabsorption was corrected using indomethacin (a cox inhibitor), forskolin (an AC activator) and cAMP replacement

(Peterson et al. 1993). These studies demonstrate that it was the elevated PGE_2 levels and not the hypercalcemia which impaired TAL function.

PGE_2 is clearly a component of the mechanism underlying this disease process. However, further studies are required which clearly establish the role of EP receptor subtypes. Also, prostanoids other than PGE_2 may be involved in the impairment of TAL reabsorptive capacity in chronic hypercalcemia. Both PGI_2 and PGE_2 play a role in inhibiting AVP-dependent water flow in the collecting duct; therefore, there may also be a role for PGI_2 that is complimentary to the action of PGE_2 in the TAL.

1.10 Rationale

EP receptor subtype characterization in rat mTAL is incomplete. There is cell signaling evidence which clearly characterizes the EP_3 receptor subtype in rat mTAL. There is also cell signaling evidence indicating the presence of a Gs protein coupled EP receptor subtype; however, it is unclear whether the cAMP responses observed are mediated by EP_2 or EP_4 receptor subtypes. This can be further understood by comparing the cAMP responses to PGE_2 alone and together with the EP_4 receptor subtype antagonist AH-23848B. While there is limited evidence suggesting the presence of the EP_1 receptor subtype, the cAMP response of PGE_2 alone and together with the EP_1 receptor subtype specific antagonist AH-6809 have not been compared to look at an inhibitory role for this receptor subtype on cellular cAMP.

Both PGI_2 and PGE_2 play a concerted inhibitory role on AVP-dependent water flow in the collecting duct. While PGE_2 also inhibits the AVP response of the mTAL, there has been no investigation of any potential role for PGI_2 in this region of the nephron. This issue can be approached using the PGI_2 analogs iloprost and cicaprost. These analogues have greater chemical and metabolic stability than PGI_2 at physiological pH.

1.11 Purpose

The purpose of this project was to complete the characterization of EP receptor subtypes and to investigate the presence of an IP receptor in the rat mTAL. This was accomplished through biochemical studies assaying cellular signaling responses and gene expression studies using in situ hybridization.

1.12 Approach

Our laboratory adopted and modified a method of quickly isolating rat mTAL in suspension (Trinh-Trang-Tan et al. 1986). Second messenger studies were done by incubating this fresh mTAL suspension with AVP and PGE_2 and PGI_2 receptor agonists and antagonists. Following each experiment, cAMP radioimmunoassay was done to determine the second messenger responses directly or indirectly. Second messenger responses were studied directly in the cases where the receptor was either Gs or Gi protein coupled to AC. Responses would be

studied indirectly if observed changes in intracellular cAMP occured through receptor coupling of Gq regulatory protein to PLC, and mediated through PKC. In these studies, changes in cAMP levels measured by radioimmunoassay might provide evidence of cell signaling cross-talk between PKC and cAMP.

The fresh preparation of rat mTAL in suspension used for these experiments was advantageous since the mTAL are quickly isolated and a viable yield of mTAL is obtained. The final mTAL suspension had a high purity at 90-95% which indicates 5-10% contamination from other tubule segments in the outer medulla. For each receptor or receptor subtype where evidence is observed at the biochemical (cell signaling) level, we looked for gene expression of the receptor in the mTAL. We were able to associate the pattern of receptor expression using in situ hybridization withTamm-Horsfall immunoperoxidase staining on serial sections from rat kidney.

Section 2: Materials and Methods

2.1 Cell Signaling Studies

2.1.01 Rat mTAL Dissection and Isolation

2.1.01.1 Preparation

1. 500ml of 10 x Hanks Balanced Salt Solution (HBSS) was prepared by adding 50ml of the HBSS concentrate and 1.19g HEPES to 450ml dH_20. The ionic millimolar composition of the diluted HBSS was 1.3 $CaCl_2$, 5 KCl, 0.3 KH_2PO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 137.9 NaCl, 0.3 Na_2HPO_4, 5.6 D-glucose and 0.3 phenol red. The medium was prepared and the pH adjusted to 7.4 prior to oxygenation for 30 minutes with a mixture of compressed 95% O_2 and 5% CO_2. The solution was split into two equal volumes and the pH of one was adjusted to 7.35 and the other to 7.45. The former was used at room temperature, while the latter for incubations at 37°C.

2. All glassware used with the mTAL suspension was silanized with Sigmacote (Sigma). This reduces the loss in tissue yield that occurs when the mTAL stick to untreated glass surfaces. The glassware that required silanization included:

- 1 x 100ml Erlenmyer flask
- 1 x 15ml test tube
- 1 x 50ml beaker

3. Prepare 1.5% bovine serum albumin (BSA) in HBSS was prepared and the pH was readjusted to 7.4.

2.1.01.2 Tissue Dissection and Isolation

The isolation of a viable suspension of rat mTAL was modified from the method developed by Trinh-Trang-Tan et al. (1986). This modified method was used in all experiments involving isolation of the rat mTAL for cAMP studies and is described in the following outline.

1. Two male Sprague-Dawley rats weighing approximately 250g were anesthetized with a 60mg/kg intra-paritoneal dose of somnitol (sodium phenobarbitol).

2. After the toe pinch reflex is no longer present, bilateral nephrectomy was performed on the animal. Using a pair of suture scissors, the abdominal cavity was opened behind the rib cage. To reduce the interference from animal hair in the dissection, the area where the skin was cut was soaked with EtOH until the fur became matted down. Once through the different skin layers, the abdominal viscera was displaced to expose the kidney. The kidney was held between thumb and forefinger (wearing gloves) and suture scissors were used to sever the kidney from the abdomen where the renal artery, vein and ureter meet the kidney. This kidney is placed in a 20ml beaker containing dissection medium (HBSS with 10^{-5} M indomethacin on ice). Following this, the second kidney on the side of the incision is quickly removed. Minimizing the time for nephrectomy was important because PGE_2 production in the intact kidney increases after the first

nephrectomy. This procedure was repeated with the second rat and the four kidneys were transferred to another 20ml beaker on ice containing fresh dissection medium.

3. The fat and renal capsule were removed from one kidney. First, an incision was made in the capsule on the inside curvature of the kidney and in a single motion, the capsule was peeled away from the kidney to the point where the only attachment between capsule and kidney remaining was where the renal vasculature enters and exits. The capsule, vasculature and ureter were severed from the kidney by cutting at this point using curved dissecting scissors. Five 1mm thick slices along the cortico-medullary axis were cut using a scalpel. It helps to maintain the kidney in position for this slicing by holding it flat on its side with a clean microscope slide. The polar ends of the kidney were discarded as they contain no appreciative amount of outer medullary inner stripe. The slices were placed in a petri dish on ice containing dissecting medium. This procedure was repeated with the other 3 kidneys.

4. Under the stereomicroscope at 12x magnification, each slice was dissected using curved and ophthalmic scissors. The inner medullary and papillary regions (white inside portion) were removed and discarded first because these regions produce high levels of prostanoids. Next, the cortical region and outer stripe of the outer medulla (brown outer stripe) were separated from the inner stripe of the outer medulla (red stripe in the middle region). The tissue from the inner stripe of the outer medulla was placed in a second petri dish on ice containing dissecting medium. The cortical tissue was discarded.

5. 50ml of 0.7% collagenase solution was prepared by adding 35mg collagenase A to 50ml HBSS containing 10^{-5}M indomethacin. This solution was kept in a 37°C H_2O bath until ready for use.

6. The dissecting medium was carefully decanted and drained from the petri dish containing the pieces of outer medullary inner stripe tissue. The tissue was then placed on and forced through a 1mm x 1mm polypropylene gauze filter with a weighing spatula to break it up into equal sized cubes. The tissue cubes were then taken off the gauze and placed in a 25ml conical polypropylene tubes containing 10ml of collagenase solution.

7. The tube containing collagenase and tissue was incubated by shaking in a 37°C H_2O bath for 10 minutes. The solution and outer medullary chunks were forced three times through a 15cm piece of PE 240 tubing attached to a 10cc syringe with a 15G luer adapter. This step required drawing all 10ml of digestion medium containing outer medullary tissue pieces into the syringe; the purpose being to subject the outer medullary tissue to the mechanical stress of passing through the PE tubing. After this first digestion, the tissue suspension was allowed to sit for 1 minute while the larger chunks were allowed to settle to the bottom. At this point, the supernatant, containing mTAL in a crude suspension, was drawn off with a 10cc syringe attached to the PE 240 tubing. Almost 10ml of supernatant was drawn off and placed in a 100 ml silanized Erlenmyer flask. To keep the mTAL from clumping, 4ml of 1.5% BSA was added and the solution kept on ice. This digestion procedure was repeated four more times with a 15cm piece of PE 190 tubing substituted for the PE 240 tubing. This series of alternating enzymatic and mechanical digestion has been optimized for the liberation of mTAL in suspension. While the

conditions are harsh in that they destroy much of the weaker tubule segments, the resiliency of the mTAL enhances their survival.

8. At the end of the five digestion periods, the crude mTAL suspension was passed through a 100μm filter mesh secured by an elastic band to the top of a 100ml beaker. The pores in this size of mesh are large enough to let individual cells and smaller tubule fragments that have escaped digestion pass, yet small enough to trap larger tubule fragments like the medullary thick ascending limb. This step serves to purify the crude suspension to about 90% mTAL.

9. The mesh was then inverted onto and fastened to a silanized 50ml beaker using the elastic band. The mTAL were gently washed off the mesh with 4ml HBSS from a 5cc syringe and 30G needle into the 50ml beaker. The mTAL suspension was then poured into a silanized 15ml test tube and kept on ice.

10. At this point, the viability of the mTAL was assessed using the trypan blue exclusion test. Using pipette tips with the ends cut to a 1mm wide opening, 10μl of mTAL suspension was placed onto a microscope slide together with 10μl of trypan blue. The proportion of viable mTAL were assessed by differentiating those mTAL which had not taken up the trypan blue stain (viable) from those which did (non-viable). The initial stage of this M.Sc. project involved practicing this mTAL isolation procedure to consistently obtain yields of 400μg mTAL tissue per four rat kidneys with less than 5% non-viable tissue as determined by trypan blue exclusion.

11. The mTAL suspension was centrifuged at 80g for 5 minutes. The supernatant was discarded and the mTAL resuspended in 2ml of pre-incubation medium. The reconstituted suspension was gently vortexed to reduce any tissue clumps that may have resulted from centrifugation. The preincubation medium consisted of HBSS with 10^{-5}M indomethacin, a cyclooxygenase inhibitor, and 5 x 10^{-5}M Ro20-1724, a phosphodiesterase inhibitor. A cyclooxygenase inhibitor was used to minimize the effect of endogenous prostanoids so we could more accurately look at the effect of prostanoids added exogenously. Since all our cell signaling studies relied on cAMP measurements, the enzyme responsible for its degradation, phosphodiesterase, was also inhibited.

2.1.02 Preparation of Samples for Cell Signaling Studies

The suspension of mTAL was distributed in 50µl aliquots in 1.5ml eppendorf tubes. The number of aliquots depended on the number of experimental conditions (agents used and concentrations) and controls used. Every condition and control was tested in duplicate and four aliquots were set aside for protein determination. The total number of experimental samples aliquoted was divided by four (the number of protein aliquots) and the number obtained was the frequency with which a protein aliquot was made when aliquoting the experimental samples. For example, if twenty aliquots were to be made for the experimental samples, one protein aliquot would be made for every five experimental samples aliquoted. Before aliquotes were pipetted for protein determinations and experiments (the pipette tip was cut so that the diameter of the opening was about 1 mm), the test tube containing the mTAL suspension was gently vortexed. In

this way , a reasonable homogeneity in mTAL concentration can be achieved for every 50µl aliquot. Using this rationale, the same value for protein content was assigned to every experimental sample, and the value for the protein content was calculated from the average of the four samples aliquoted for protein assay.

2.1.03 Protein Determination

The Biorad protein assay, a modification of the Bradford method, was used to measure protein content throughout these studies (Bradford 1976). The assay involves adding coomassie blue dye to hydrolyzed proteins. The absorbency maximum of the dye changes from 465nm to 595nm when basic and aromatic amino acids are bound and a colour change occurs that is proportional to the amount of protein it binds. The 50µl aliquots for protein determination are centrifuged at 2000g for five minutes and the supernatant was discarded. The mTAL pellet was then resuspended in 150µl 1N NaOH. These samples were then left to digest into soluble protein overnight at room temperature before being assayed for protein content. The following day, the protein samples were centrifuged at 1500g for 5 minutes, and 50µl was aliquoted into 750µl distilled H_2O in a test tube. To this, 200 ul of the Biorad protein assay dye reagent was added and the test tube was well vortexed. A water blank consisting of 800µl dH_2O plus 200µl dye reagent was made and used to "blank" the spectrophotometer (Beckman). Also, since NaOH, the agent used to solubilize the proteins, interacts with the dye and interferes with the assay, a NaOH blank was made consisting of 750µl H_2O, 50µl 1N NaOH and 200µl dye reagent. The value obtained

for this NaOH blank was subtracted from each of the four values for the protein samples. The standard concentrations were 1.56, 3.12, 6.25, 12.5 and 25ug/ml of BSA in 800µl dH_2O such that when 200µl of the dye reagent was added, the final concentrations were 1.25, 2.5, 5.0, 10.0 and 20.0µg/ml. The dye reagent was allowed to incubate with the protein solution for at least five minutes and no longer than thirty minutes. The samples, standards and blanks were transferred to 1ml cuvettes and their absorbencies at 595nm were measured using a Beckman spectrophotometer. A linear regression standard curve was generated from the optical density of the five standard concentrations at 595nm (OD_{595}) plotted against the protein values in ug using the Microsoft Excel software package. Protein content for the unknown samples was determined by comparing their absorbencies to the standard curve and interpolating the value for protein content.

2.1.04 AVP Dose Response Curve

Arginine Vasopressin (AVP) increases intracellular cyclic AMP by stimulating the V_2 receptor subtype which is coupled to AC through the Gs protein in the rat mTAL. Therefore, to test functional viability of our suspension of mTAL we established a dose curve response to AVP concentrations in the acceptable physiological range from 10^{-10}M to 10^{-6}M. The protocol we used was a modification of the one developed by Trinh-Trang-Tan et al. (1993). Serial concentrations of AVP were made in HBSS from which 50µl would be taken and added to their respective eppendorfs to bring those samples to the required concentration of AVP. Using an aqueous solution of AVP from Sigma chemical company, serial dilutions of AVP were made in

HBSS starting with 2 x 10^{-6}M AVP in a total volume of 500µl in 1.5ml eppendorf tubes. 50µl of this solution was added to 450µl HBSS, making the AVP concentration in that tube 2 x 10^{-7}M. Serial dilutions were made in this manner until the final concentration of 2 x 10^{-10}M was obtained. 50µl of medium containing 2 x 10^{-6}M AVP was added to 50µl of aliquoted mTAL suspension such that the final volume of 100µl would contain an AVP concentration of 10^{-6}M. All serial concentrations of AVP were prepared in preincubation medium and kept on ice until the beginning of the experiment. In all experiments involving cAMP measurements where exogenous agents were added, the agents were first prepared in HBSS media at concentrations which when added to the 50µl mTAL aliquots, would bring the total volume to the desired concentration under study.

Sixteen 50µl samples of mTAL were aliquoted into eppendorf tubes. Four tubes were used for protein determination and 12 for duplicates of the control and each concentration of AVP tested. Each experimental condition was run in duplicate. The samples of mTAL suspension were kept on ice until the beginning of preincubation, which took place in a 37°C water bath for 10 minutes. After preincubation, the appropriate concentration of AVP was added for an incubation period of 10 minutes at 37°C. To stop the reaction, the samples were placed in a dry ice-acetone slush. After the experiment, the samples were treated to remove intracellular cAMP from within the cells using a modified freeze-thaw technique adapted from previous studies (Trinh-Trang-Tan et al. 1993). The samples were heated in an 80°C water bath for 15 minutes before 400µl of ice cold 5% formic acid in EtOH was added to deproteinize the membranes. The tubes were then shaken vigorously in an ice-water bath for a further 15 minutes. The samples were then centrifuged at 200g for 10 minutes to precipitate cellular and subcellular components.

400µl of supernatant was carefully pipetted into fresh 1.5ml eppendorf tubes. The samples were evaporated in a vacuum dryer at 80°C and reconstituted in 300µl K_2HPO_4 buffer at pH 6.2. This allowed the extracted cAMP product to be stored overnight at -86°C for assay the following day, without significant cAMP degradation. This procedure was employed for all experimental studies measuring changes in cAMP.

2.1.05 Stimulation of cAMP with Prostanoids and Receptor Subtype Specific Agonists

The cAMP dose-response was tested to varying concentrations of PGE_2, the EP_2 receptor subtype specific antagonist butaprost and the PGI_2 analogs iloprost (ILP) and cicaprost (CCP). The doses tested for all these agents were in ten-fold increments from 10^{-11}M to 10^{-5}M. The preincubation period was similar to the AVP dose-response experiments at 10 minutes, but the incubation period was 20 minutes in duration. The incubation period was longer because of the suspected weaker cyclic AMP response to these agents. Following the incubation period, the samples were frozen in a dry ice/acetone slush and processed as described above.

2.1.06 Prostanoid Dose-Response Inhibition of AVP-Dependent cAMP

Previous studies using PGE_2 show this prostanoid to inhibit AVP-dependent cyclic AMP in the rat mTAL. This inhibitory response was tested in our mTAL preparation, as was a possible

likewise response to the PGI_2 analogs ILP and CCP. The dose range tested for ILP and PGE_2 was 10^{-11}M to 10^{-6}M in ten-fold increments and for CCP 10^{-11}M to 10^{-5}M. Increasing doses were used until an observed inhibitory effect was maximal or the dose was no longer within the physiological range. The samples were allowed to preincubate for 10 minutes before incubation with the prostanoid or analog. Following this, the mTAL preparation was incubated for 10 minutes with 10^{-7}M AVP and a maintaining dose of the prostanoid or analog. Various sequences of incubation were tested: AVP followed by prostanoid, coincubation of both AVP and prostanoid, and prostanoid followed by AVP. The AVP dose used, 10^{-7}M, was that which approached sub-maximal levels of cAMP from the AVP dose-response curve. The rationale behind using a near maximal AVP dose was to provide a large window for inhibition of AVP-dependent cyclic AMP. Following the timed incubations, the reaction was terminated and cyclic AMP was measured.

2.1.07 Additivity Experiments

Unlike PGE_2, ILP and CCP did not stimulate cAMP generation. However, the two PGI_2 analogs did attenuate AVP-dependent cAMP production. Therefore to establish the presence of different receptors, experiments testing the additivity of inhibition of AVP-dependent cyclic AMP by PGE_2 and ILP, and PGE_2 and CCP were conducted. While the lack of additivity does not necessarily mean that the prostanoids act on the same receptor, observed additivity would establish two distinct receptors. The concentrations of PGE_2, ILP and CCP chosen were those which caused maximal inhibition of AVP-dependent cyclic AMP production. The concentrations

used for PGE_2 and ILP were 10^{-8}M and 10^{-7}M respectively. In the experiments, the sequence of addition bears potential significance because in the study by Hébert et al. 1995 it was shown that PGE_2 can bind to but not activate putative IP receptor subtypes in the rabbit cortical collecting duct. Therefore, following a 10 minute preincubation and prior to a 10^{-7}M AVP incubation at 37°C, the mTAL aliquots were incubated for 10 minutes with ILP before addition of PGE_2 with sufficient ILP to maintain the dose of the PGI_2 analog. The incubation reactions were stopped by freezing mTAL samples in a dry ice/acetone slush before work up of the cAMP product was carried out.

2.1.08 Butaprost Stimulation

Increasing concentrations of PGE_2 caused increased cAMP production, indicating stimulation of AC through Gs protein coupling. There are two different EP receptor subtypes which elevate cAMP through this mechanism: EP_2 and EP_4. Therefore cAMP production in response to doses of the EP_2 receptor subtype specific agonist butaprost from 10^{-11}M to 10^{-5}M was measured. The mTAL aliquots were pre-incubated for 10 minutes before being incubated with butaprost for 20 minutes at 37°C.

To examine the specificity of butaprost for the EP_2 receptor subtype, the cAMP response from 10^{-10}M to 10^{-5}M butaprost was also tested in the presence of 10^{-7}M AVP. This test would look at any butaprost action on the EP_3 receptor subtype that would attenuate AVP-dependent cAMP. After pre-incubation, the mTAL samples were first incubated with butaprost prior to

incubation with AVP and a maintaining dose of butaprost at 37°C. In both sets of butaprost experiments, the mTAL samples were processed and cAMP was were stored overnight at -86°C.

2.1.09 Mechanism of Action

The mechanism of action behind prostanoid receptor mediated inhibition of AVP-dependent cyclic AMP, was examined in two sets of experiments. One set of experiments looked at the role played by the G inhibitory (Gi) protein in attenuating AVP-dependent cyclic AMP levels through the action of the Gi protein inhibitor pertussis toxin (PTX). The other set of experiments looked at the potential role the EP_1 receptor subtype and IP receptor play in inhibiting AVP-dependent cAMP through protein kinase C (PKC) cross-talk. Part of the PGE_2, ILP and/or CCP mediated inhibition of AVP-dependent cAMP may be G regulatory protein-coupled to phosphatidyl inositol hydrolysis. Through cell signaling cross-talk, PKC has been shown to attenuate cyclic AMP in other experimental models. Two PKC inhibitors, bisindolylmaleimide-1-hydrochloride (BIS.1) and calphostin C, were used to test the potential involvement of PKC and were specifically chosen for their specificity of inhibition or PKC. Staurosporine, a more traditional PKC inhibitor, is less specific and has been shown to inhibit other protein kinases such as protein kinase A and myosin light chain kinase.

2.1.09.1 Pertussis Toxin Experiments

The two major parameters that had to be worked out for the experiments involving PTX were the concentration and duration of preincubation required. PTX pretreatment at 37°C can not be too long because the mTAL viability will diminish with time. This gradual tissue death is due to the fact that although the media is saturated with O_2 prior to experiments, the 50µl mTAL aliquotes are not oxygenated during preincubation or incubation stages. Over time and especially at the higher temperature of 37°C, dissolution of O_2 from the media will occur. The optimal concentration of PTX also had to be determined. Some studies use 500ng/ml for prolonged pretreatment periods lasting two hours and longer (Milligan 1988, McKenzie 1992). However, 1ug/ml PTX has been used for short duration pretreatments, but cytotoxicity can be a problem with higher doses of PTX. Therefore pretreatment periods of 60 and 120 minutes and concentrations of 500ng/ml and 1ug/ml were tested to establish a compromise that maximized tissue viability and minimized PTX cytotoxicity. After analyzing preliminary results, it was decided to pretreat with 1ug/ml PTX for 60 minutes. After this pretreatment period, the mTAL were incubated with either 10^{-8}M PGE_2, 10^{-7}M ILP or 10^{-6}M CCP (the concentrations for which maximal inhibition of AVP-dependent cAMP was observed for 10 minutes). The mTAL were then incubated with 10^{-7}M AVP and a maintaining dose of prostanoid or analog for a further 10 minutes before stopping the reaction. A control testing the functional viability of the mTAL was done by comparing the mTAL response to 10^{-7}M AVP above basal values before and after 60 minutes, the time allotted for PTX pretreatment. Other controls included testing the effect of PTX treatment alone and the effect of heat inactivated Ptx (PTX treated for 2 hours at 60°C) on PGE_2, ILP and CCP mediated inhibition of AVP-dependent cAMP.

2.1.09.2 Inhibition of PKC:

Bisindolylmaleimide

BIS.1 pretreatment for 30 minutes in bum concentrations was used to study the potential role of PKC. Medium containing BIS.1 was pipetted into the mTAL aliquotes for 30 minutes pretreatment prior to the mTAL incubation for 10 minutes with 10^{-8}M PGE_2, 10^{-7}M ILP or 10^{-6}M CCP. Following this, the mTAL were incubated with 10^{-7}M AVP for a further 10 minutes before stopping the reaction. Controls for these experiments included testing the AVP response above basal values after 30 minutes, testing for an inhibitory action of phorbol myristate acetate (PMA, a PKC activator) on AVP-mediated cAMP, testing for an effect of BIS.1 on possible PMA-mediated attenuation of the AVP-dependent cAMP, and testing for an effect of BIS.1 on possible AVP-dependent changes in PKC-mediated cAMP levels.

Calphostin C

mTAL were pretreated with 10^{-7}M calphostin C for 10 minutes. Long pretreatment periods and concentrations greater than 1uM had to be avoided when using this PKC inhibitor because of its cytotoxic effects. The mTAL were allowed to preincubate for 10 minutes in a 37°C water bath before medium containing calphostin C was added. After 10 minutes calphostin C

treatment, the mTAL were incubated with 10^{-8}M PGE_2 or 10^{-7}M ILP prior to incubation with 10^{-7}M AVP for a further 10 minutes. Controls for these experiments were similar to those for the BIS experiments with the exception that the AVP response above basal values was tested after 10 minutes preincubation only and calphostin C was used in place of BIS.1 with PMA.

2.1.10 PGE_2 Receptor Subtype Specific Antagonists

The possible role of the EP_1 receptor subtype in mediating the inhibition of AVP-dependent cyclic AMP observed with PGE_2 and ILP was further studied using the EP_1 specific antagonist AH-6809 from Glaxo-Wellcome in an attempt to partially block this inhibitory effect. The mTAL samples underwent a 10 minute preincubation prior to pretreatment with 10μM AH-6809 for 10 minutes in a 37°C water bath. The mTAL were then incubated with 10^{-8}M PGE_2 or 10^{-7}M ILP and a maintaining dose of AH-6809. The reason a possible blocking action of AH-6809 for ILP mediated inhibition of AVP-dependent cAMP was tested is because ILP is known to be a weak EP_1 receptor subtype agonist. Following PGE_2 or ILP incubation, the mTAL were incubated with 10^{-7}M AVP and maintaining doses of AH-6809 and PGE_2 or ILP. The controls used were the cAMP response to 10^{-7}M AVP over basal values, the inhibition of AVP-dependent cAMP with both PGE_2 and ILP, and the effect of AH-6809 on AVP-dependent cAMP values to test drug cytotoxicity.

The cAMP generating response of the mTAL to both PGE_2 and BUT was further investigated with respect to differentiating the role of EP_2 and EP_4 receptor subtypes. BUT increased cAMP levels, indicating the presence of an EP_2 receptor subtype. However, the cAMP

response to BUT was less than that observed for PGE_2. This may or may not imply a putative role for the EP_4 receptor subtype because although the cAMP response to BUT is less, the potency of BUT for the EP_2 receptor subtype is weaker than that of PGE_2 (Coleman et al. 1994). The response of cAMP to 10^{-6}M PGE_2, the concentration where near maximal cAMP response was observed and compared with and without the EP_4 receptor subtype antagonist AH-23848B from Glaxo-Wellcome. The mTAL were allowed to preincubate for 10 minutes prior to the addition of medium that would bring the AH-23848B concentration to 10μM for 10 minutes. Medium was then added bringing the final PGE_2 concentration to 10^{-6}M and maintaining the dose of AH-23848B. As controls, the cAMP response with and without AH-23848B was also measured for 10^{-7}M AVP to test cytotoxic effects, 10^{-6}M BUT and 10^{-6}M forskolin (FSK) to test possible drug interactions with adenylate cyclase.

2.1.11 cAMP Radioimmunoassay

The cAMP content of the samples was assayed using a radioimmunoassay kit obtained from Diagnostic Products Corporation. The endogenous cAMP product competes with a known amount of ^{3}H-cyclic AMP for a known amount of binding protein. The greater the amount of endogenous cAMP present, the less ^{3}H-cAMP will be bound. The unbound cAMP is removed by adsorption to dextran-coated charcoal. The results are quantitated by counting the samples in a liquid scintillation counter and comparing the counts generated by the experimental values with those on a standard curve. The cAMP range for the standard curve is 0.11, 0.33, 1.00, 3.00, 9.00, 27.00 picomoles. The protein bound counts are a function of the unlabelled cAMP values. The

cyclic AMP standard curve was generated by using the Graph Pad Prism software package to plot a one-phase exponential decay, non-linear regression analysis from the counts obtained for the calibration standards. The software package can then compare the counts obtained from the unknown experimental samples to the standard curve and interpolate the cAMP content for these samples.

2.2 Immunoperoxidase Staining and In Situ Hybridization

The mTAL isolation procedure described above is advantageous in many ways: it is relatively quick and provides a viable suspension of rat mTAL in good yield. Although the purity of the final suspension is high at 90-95%, this does present a drawback when studying second messenger responses. Five to ten percent contamination does allow for the possibility that some of the cAMP responses observed and possibly all of a weak response may be attributable to non-mTAL segments. The EP_1, EP_2 and EP_3 receptor subtypes and a putative IP receptor have all been characterized in the collecting duct. Also, the EP_2, and/or the EP_4 receptor subtype(s) may be present along the thin descending limb as PGE_2 has been shown to generate cAMP in this tubule segment in the rat (Breyer 1995). Therefore, possible contributions to cAMP responses from these tubule segments can not be ignored.

For this reason, other studies were done to characterize receptors for which there is second messenger evidence in the mTAL. Ideally we would like to use receptor subtype specific antibodies and colocalize them with antibody markers for the mTAL marker Tamm-Horsfall glycoprotein. However, after an exhaustive search, no PGE_2 receptor subtype specific or PGI_2

receptor specific antibodies were found that were commercially available or in literature reviews. All of these receptors have been cloned, which therefore makes it possible to colocalize expression of the receptor genes together with the Tamm-Horsfall (TH) glycoprotein using in situ hybridization and immunohistochemistry. Only expression of the EP_3 receptor subtype gene has been shown in the mTAL using in situ hybridization for that receptor subtype colocalized with TH glycoprotein immunoperoxidase staining in rabbit, mouse and human kidney sections (Breyer et al. 1993, Sugimoto et al. 1994, Breyer et al. 1996). We therefore decided to attempt this colocalization through simultaneous detection of EP_3 mRNA and TH glycoprotein on the same rat kidney tissue sections. Once we had established a working procedure for EP_3 receptor subtype mRNA, we would do the same for the other E-prostanoid receptor subtypes and I-prostanoid receptor for which second messenger evidence was present.

The order in which to conduct these studies (immunostaining and in situ hybridization) had to be determined, keeping in mind that conditions for in situ hybridization can destroy unstable antigen and that any RNase contamination introduced by the immunostaining procedure can destroy the sought after mRNA. Given that TH is a relatively stable glycoprotein and not being certain of the presence of RNases in the antibody solutions or serum used for immunostaining, we tried doing the in situ hybridization first followed by the immunostaining. Although the in situ hybridization worked well, the immunostaining did not. We then tried reversing the procedure with immunostaining followed by in situ hybridization. The immunostaining worked; however, the in situ x-ray autoradiography results were suspect because for both the EP_3 sense and anti-sense probes there was intense labeling on the areas of kidney section staining TH-positive. Both EP_3 probe and its control were binding non-specifically to the

diamino benzidine (DAB) stain. Also, there appeared to be no hybridization of the EP_3 probe to TH kidney sections where there was no DAB stain. Contamination by RNases may have destroyed the endogenous mRNA. The following modifications were made based on a literature search and consultation with Dr. Matthew Breyer's research group which has published similar work on rabbit and human kidney slices (Brahic et al. 1984, Barnes et al. 1987). To eliminate the problem of non-specific binding of probe to diaminobenzidine (DAB) stain, I added an acetylation step between the immunostaining and the in situ hybridization using triethanolamine (TEA) and acetic anhydride. The amine groups on the DAB stain are nucleophilic and will bond to the DNA oligo probes, resulting in non-specific hybridization. The acetylation reaction converts these amine groups to amide groups, rendering them unreactive. To eliminate possible RNase contamination in the immunostaining procedure, the RNase inhibitor RNasin at 100 u/ml (Promega) was added to all antibody and serum dilutions. RNasin binds with and inhibits RNase in a 1:1 molar ratio. Incorporating these two steps solved the problems of non-specific binding of the radioprobe to the DAB stain, and eliminated RNase contamination. However, the resulting colocalization with TH immunostaining was unconvincing.

We were unsuccessful in trying to colocalize TH immunoperoxidase staining for the EP_3 receptor subtype mRNA on the same tissue section. We therefore resorted to TH immunostaining on one section and in situ hybridization on the adjacent section. If we could show similarity in outer medullary tubule pattern between serial sections of rat kidney, one showing in situ receptor expression and the other TH immunoperoxidase staining, we could associate expression for that receptor along the mTAL. While we were at a loss in being unable to show receptor expression together with TH immunostaining along the same mTAL, we were at

an advantage in not having to meet the difficult compromises necessary to colocalization on the same tissue sections. The individual procedures for TH immunoperoxidase staining and in situ hybridization are described as follows.

2.2.1 Immunoperoxidase Staining

The immunoperoxidase staining procedure was done using commercially available goat antiserum to human uromucoid, Organon Teknika Corporation and the Vectastain ABC kit from Vector laboratories. The frozen kidney sections were fixed with 10% buffered formalin for five minutes at 4°C, followed by two one minute PBS washes. At this point, the slides were immersed in 0.3% H_2O_2 in MeOH for 30 minutes in the dark to inhibit endogenous peroxidase activity in the tissue sections, which helps reduce background peroxidase staining. Prior to incubation with primary antibody, the tissue sections were incubated with 100µl of 0.15% rabbit serum in PBS to prevent non-specific binding between the secondary antiserum (rabbit anti-goat IgG, Vector laboratories) and rat kidney tissue. The tissue sections were then incubated with 100µl from a 1:200 dilution of primary antibody. Control sections were done using the same dilution of non-immune goat serum. The slides were washed in PBS for 10 minutes and the tissue sections were incubated with 100µl from 2ug/ml biotinylated secondary antibody for 30 minutes at room temperature. The unbound secondary antibody was washed from the tissue sections with PBS for 10 minutes and an avidin-horseradish peroxidase complex was added to the sections.

Avidin DH was complexed with biotinylated horseradish peroxidase H in dilute solution for 30 minutes before 100µl was pipetted onto the blotted tissue sections. A 60 minute

incubation time was allotted for this complex to bind with the secondary antibody through the biotin-avidin interaction. Avidin is a glycoprotein with very high affintiy (10^{15}/M) for the vitamin biotin. This affinity is over one million times greater than the interaction between most antibodies and antigens and makes the avidin-biotin binding essentially irreversible. Macromolecular complexes can be formed when enzymes are conjugated with biotin because avidin has four biotin binding sites. The tissue sections are again washed in PBS for 10 minutes and carefully blotted before 100µl of DAB and H_2O_2 (Sigma Fast DAB Peroxidase Substrate Tablet Set, Sigma) are pipetted onto them. The peroxidase previously added reacts with the H_2O_2 to produce O_2 and H_2O. The O_2 then reacts with the DAB to produce an insoluble black-brown precipitate. The peroxidase-DAB reaction was allowed to proceed until the appearance of the brown stain was observed with the naked eye, which took about 5 minutes. This reaction was stopped by washing the slides in tap water for 5 minutes. The tissue sections were counterstained with eosin, washed and mounted to coverslips using Permount (Sigma).

2.2.2 In Situ Hybridization

Throughout the in situ procedures, sterile conditions were observed to eliminate sources of RNase contamination. All glassware, weighing spatulas and stir bars were baked overnight at 160°C. All solutions should be made with diethylpyrocarbonate (DEPC)-treated dH_2O and autoclaved. All tissue is handled with autoclaved surgical instruments and kept in autoclaved, DEPC-treated phosphate buffered saline (PBS) at pH 7.4. One litre of DEPC-treated-PBS contains 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4. One ml of DEPC is added to

the solution and allowed to mix for at least 3 hours in the fume hood. The solution is also autoclaved before use.

2.2.2.01 Choosing the Nucleotide Sequence for the DNA Oligo Probes

DNA sequences for the prostanoid receptor subtype mRNAs being studied were down loaded from the GenBank Database of the National Centre for Biotechnology Information (NCBI) web site and analyzed with the Oligo software package. Oligo probes 50 nucleotide sequences in length were chosen that had minimal repetitive sequences that could contribute to non-specific hybridization and no secondary nucleic acid structures which could interfere with the probe binding to the endogenous mRNA. The oligo probes were not chosen from regions of the mRNA containing homooligomers, dinucleotide sequence repeats, hairpin loop and duplex structures. Also, sequences were chosen with a guanine-cytosine (GC) nucleotide content of about 55%. Guanine and cytosine hybridize with each other through three hydrogen bonds. Therefore those two nucleotides hybridize more strongly than adenosine and thymidine/uracil which share only two hydrogen bonds. If the GC content of the probe is too low, the probe may be removed from the target mRNA in post-hybridization washes. If the GC content is too high, the probe may bind non-specifically to the GC portions of other mRNA sequences.

Once a tentative probe has been picked, the uniqueness of the sequence and no homology with other rat mRNA had to be verified. To do this, the sequence was sent to the GeneBlast service at NCBI via e-mail which compared each sequence with that of every cloned gene. The returned results displayed the percentage of sequence homology the analyzed sequence shared

with other mRNA. Only sequences which bore no homology to any reported mRNA present in the rat kidney were chosen.

. All DNA oligo probes were ordered from the University of Calgary University Core DNA Services. The synthesis scale chosen was 0.2umole and the probe was purified by gel electrophoresis. The sequence ordered was complimentary to and the reverse order (5' to 3') of the endogenous mRNA. Together with the probe, a control 50-mer oligonucleotide of identical sequence and order to the endogenous mRNA was also ordered. The control oligo can not bind to the target mRNA and represents the background level of hybridization. The following table presents the nucleic acid sequences of the probes used.

Prostanoid Receptor Subtype	mRNA Sequence Chosen for Oligonucleotide Probe (5' to 3')
EP_3	GACGCCGGGAGAGCAAACGCAAAAAGTCTTTCCTGCTGTGCATTGGCTGG
EP_2	TTTGGAGCTCACGTTCGATCTAGGGATGGTATCTGTAGGGTGGGGTGCG
IP	CCCACGGTGGGACGATGCTGTGTGACACTTTCGCCTTCGCTATGACTTTC

2.2.2.02 Tissue Sectioning

The kidney was cut longitudinally along the mid-section with a sterile razor blade. The half-kidney was then mounted onto a 3cm x 2cm card using cryobed compound (Bright Instruments) on the uncut, curved side of the kidney. The tissue was then carefully frozen using CO_2 powder, wrapped in sterile aluminum foil three times and stored at -80°C.

Frozen sections were prepared using a cryostat at -20°C (necessary to prevent tissue fracturing during slicing) and 12um thick slices are cut. The card with half-kidney was mounted

to the cryostat using cryobed compound on the side of the card opposite the kidney. The kidney was then trimmed until complete sections were consistently produced. Before making complete tissue sections, four fiduciary marks were made in the kidney along the cortical-medullary border using a sterile 26G needle. Complete tissue sections were transferred to slides (Superfrost Plus, Fisher) by thaw-mounting. Slides were then stored in a box with desiccate (drierite) at -86°C until tissue fixation.

2.2.2.13 Hybridization Buffer

The hybridization buffer was composed as follows:

- 5 ml 100% formamide
- 2 ml 20x SSC
- 0.5 ml 0.5 M sodium phosphate
- 0.1 ml 0.1 M sodium pyrophosphate
- 1 ml 50x Denhardt's solution
- 0.2 ml acid-alkali hydrolyzed salmon sperm DNA (10mg/ml)
- 0.2 ml polyadenylic acid (5mg/ml)
- 10 ul heparin (120mg/ml)
- 1g dextran sulfate, sodium salt

Note: the volume was adjusted to 10 ml with DEPC-dH_2O

2.2.2.04 Tissue Fixation

The slides were placed in a Coplin jar containing 10% buffered formalin at 4°C for 5 minutes. After 2 PBS washes for 1 minute each, the slides were dehydrated in 70% EtOH for 5 minutes and 95% EtOH for 1 minute. The slides were air dried for at least 30 minutes. Solutions were made using DEPC-treated dH_2O. DEPC-treated dH_2O is made by adding 1ml DEPC per litre of dH_2O. The treated H_2O is allowed to stir for at least 3 hours in a fume hood before being autoclaved.

2.2.2.05 Probe Labeling

This step involves enzymatic transfer of radiolabelled nucleotide to the oligonucleotide probe. The probe was reconstituted in sterile water to an initial concentration of 2ug/ul. This was done by first determining the total amount of probe in ug from the optical density:

#ug oligo = ODU x 33ug/ODU.

Once this was determined, enough DEPC-treated dH_2O was added to give 2 ug/ul:

#ug oligo/2ug/ul = #ul to be added.

The oligo was further diluted:

2 ug/ul → 1ug/5ul (5ul + 45ul DEPC-dH_2O) → 100ng/ul (5ul +5ul DEPC-dH_2O)

The nucleotide transfer reaction mixture was composed as follows:

- 7 ul DEPC-dH_2O
- 5 ul 5x tailing buffer

- 1 ul deoxyoligonucleotide probe (100 ng/ul)
- 10µl ^{35}S-dATP (125uCi)
- 2 ul terminal deoxynucleotidyl transferase (TdT), taken from storage at -20°C only at time of use and replaced immediately after

After adding each component, the solution was mixed by gentle vortexing and/or repeated drawing with a pipette. The terminal deoxynucleotidyl transferase (TdT) is not vortexed due to its high instability. The reaction was incubated for 2 hours at 37°C.

2.2.2.06 Probe Purification

- A Nensorb 20 column was set up in a vertical fashion, the side was gently tapped until there were no air pockets in the column matrix at the bottom.
- The cap was removed and 3ml of absolute MeOH was pipetted into the column. The cap was replaced and the MeOH was forced through the column matrix using a 10cc syringe. This was stopped by removing the cap as the fluid level approached 2-3mm above the column matrix. It was very important not to allow air into the matrix resin beyond this point.
- 3ml 0.1 M tris-HCl (pH 8.3) was added to the column and forced through with a syringe. The tris-HCl does not pass through the column matrix as easily as the MeOH.
- For each oligonucleotide, four eppendorf tubes were set up to collect drops. 500µl 0.1M tris-HCl was added to the 25ul mixture containing the labeled probe. The oligo solution was mixed by gentle vortexing and drawing repeatedly with a pipette and the 525ul was added to

the column. This was forced through the matrix until the fluid level is again 2-3mm above the column matrix. The drops were collected to the first eppendorf tube.

- The column was washed with 1.5ml 0.1M tris-HCl and this volume was collected in the second eppendorf tube.
- The oligonucleotide was eluted by adding 0.5ml 25% EtOH to the column. The first 3 drops were collected into the second eppendorf tube, and 12 drops (approximately 200µl) into the third eppendorf tube. The remaining volume was discarded into the fourth eppendorf tube.

2.2.2.07 Hybridization

First, 3 ul of the oligonucleotide were counted in 3 ml scintillation fluid using a liquid scintillation counter. The oligos were diluted with hybridization buffer to give 500ml at 5000 CPM/ul. A dilution ratio (DL) is calculated as follows:

DL = # CPM oligo - # CPM background/3ul/5000CPM/ul

volume of oligonucleotide required = 1/DL x 500µl

volume of hybridization buffer = 500-volume of oligonucleotide

Next, 16ul 3M dithiothreitol (DTT) was added to the counted probe such that the final DTT concentration was approximately 200mM. The oligos were mixed by gentle vortexing. On each tissue section, 100µl of oligo probe diluted with hybridization buffer to 5000 CPM/ul was pipetted. The oligo/hybridization buffer was pipetted onto cover slips. The fixed kidney sections were then inverted and placed upright on the coverslip until contact is made between the oligo/hybridization buffer and the tissue section. The slides were then carefully placed into the

humidity chamber with the bottom lined with tissue paper soaked in 50% formamide and 1 x SSC solution. The slides in the humidity chamber were then incubated at 45°C for 18 hours.

2.2.2.08 Washing

The unhybridized oligos were washed from the tissue sections. Using a Coplin jar, the following solutions were used to wash unhybridized oligos in the following order:

- 1x SSC (shake gently to remove the cover slips)
- 1x SSC (10mM DTT) at RT for 20 min.
- 1x SSC (10mMDTT) at 55°C for 45 min.
- 1x SSC at RT for 10 sec.
- 0.1x SSC at RT for 10 sec.
- dH_2O at RT for 10 sec.
- 70 % EtOH at RT for 10 sec.
- 95 % EtOH at RT for 10 sec.
- air dry for 30 min.

2.2.2.09 Low Resolution X-Ray Autoradiography

X-ray autoradiography is useful to localize expression of a particular receptor gene to a particular region of the kidney, for example the outer medulla. Both sides of an x-ray cassette were lined with filter paper, and the slides were secured to one side with tape. In a dark room, one x-ray film was placed over the slides such that the emulsion side of the film was in contact with the tissue sections and the cassette was closed. The x-ray was exposed for 1-7 days depending on the level of expression expected for the mRNA being investigated. After exposure, the x-ray was developed in the Radiology Department at the Ottawa General Hospital.

2.2.2.10 High Resolution Silver Emulsion Autoradiography

To obtain high resolution localization of a particular mRNA to a specific nephron segment, for example the TAL, silver emulsion autoradiography was used. The entire procedure up to and including the washing step following treatment of the slides with developer and fixer must take place in the dark room.

- 6ml of silver emulsion (NTB2, Kodak) was heated in a 42°C H_2O bath for 30 minutes, then diluted 1:1 with 6ml dH_2O. This gel-water solution was mixed very gently to prevent formation of froth or bubbles. The mixture was allowed to sit undisturbed at 42°C for a further 30 minutes.

- The gel-water mix was poured into the dipmiser slide dipping jar and left to sit in the H_2O bath.
- Each slide was dipped into the Dipmiser jar containing diluted gel 3 x 5 seconds, the back was wiped clean with tissue paper and the slide stood upright to drain and dry for 2 hours in a dark environment.
- The slides were stored in a slide box containing drierite desiccate and wrapped in aluminum foil in a refrigerator for 1-4 weeks, depending on the level of gene expression expected.
- The slides were dipped in developer 5 minutes, washed with dH_2O for 1 minute and dipped in fixative for 10 minutes. The slides were then washed in running dH_2O for 30-60 minutes. Subsequent work did not require a dark room.
- The nuclei in the tissue sections were counterstained with eosin-hematoxylin.
- After the washing/dehydration step using EtOH, the slides were immersed twice in xylene for 7 minutes each.
- The slides were allowed to air dry and are the mounted onto coverslips using permount mounting medium.

Section 3: Results

3.1 Cell Signaling Studies

3.1.1 cAMP Generating Responses

The cAMP generating response of the rat mTAL to AVP through the V_2 receptor subtype is well documented (Morel et al. 1987, de Rouffignac et al. 1991, Breyer et al. 1996). Since the cell signaling studies of this project depend entirely on cyclic AMP measurements, it was deemed necessary to examine the AVP dose-response of the rat mTAL preparation used for these studies (fig. 1). Establishing a reproducible AVP dose-response curve from the rat mTAL preparation would clearly establish the existence of a valid biological experimental model from which reliable experimental data could be derived. A cAMP dose-response curve was obtained with detectable but statistically insignificant response at 10^{-10}M AVP, and a maximal response of 366.6 $\pm$ 38.9 fmol/ug, seven fold greater than basal values, observed at 10^{-6}M AVP. AVP concentrations greater than and equal to 10^{-8}M AVP produced elevations in cAMP that were significantly greater than basal values (ANOVA, Bonferroni t-test). These cAMP measurements are consistent with previous work (Trinh-Trang-Tan et al. 1993) using the same preparation technique of rat mTAL tissue. These results made us confident that reliable and reproducible cAMP results could be generated and measured through radioimmunoassay from our mTAL preparation.

It is known that PGE_2 alone will stimulate cAMP in rat mTAL (Torikai and Kurokawa 1983, Nakao et al. 1989). While this evidence suggests a potential role for the EP_2 and/or EP_4 receptor subtypes, no further work has been done to further characterize this cAMP response to

PGE_2. the following studies were performed to reproduce the PGE_2-dependent cAMP response to determine if it is mediated via EP_2 and/or EP_4 receptors in the rat mTAL suspension. The cAMP-producing responses of PGE_2 and the EP_2 receptor subtype specific agonist butaprost (BUT) were examined(fig. 1). The dose-response curve for PGE_2 was sigmoidal with a threshold response occurring above 10^{-8}M. Maximal values of 274.5 ± 47.6fmol/ug cAMP were eleven fold greater than basal and were achieved at 10^{-6}M PGE_2. Micromolar concentrations of PGE_2 produced cAMP values significantly greater than basal (ANOVA, Bonferroni t-test). Since both the EP_2 and EP_4 receptor subtypes are coupled to cAMP generation through a stimulatory guanine nucleotide regulatory binding (Gs) protein, cAMP responses to the EP_2 receptor subtype specific agonist BUT were studied to characterize the PGE_2-dependent cAMP as EP_2 or EP_4-dependent. A threshold cAMP response was observed above 10^{-7}M BUT; however, the maximal response could not be ascertained. The response curve showed no signs of a plateau effect at 10^{-5} M BUT where cAMP values 8.3 fold greater than basal at 397.5 ± 77.3 fmol/ug were attained. It may be that this high dose of BUT is non-physiologic, making the response observed more of a pharmacological effect. The cAMP response to BUT is greater than the maximal response observed for PGE_2, and the potency of BUT for the EP_2 receptor subtype is reported to be ten fold weaker than that of PGE_2. Micromolar concentrations of BUT produced cAMP values significantly greater than basal (ANOVA, Bonferroni t-test).

To validate the EP_2 receptor subtype specificity of BUT, its effect on AVP-dependent cAMP was tested(fig.2). Inhibition of AVP-dependent cAMP would suggest that BUT has EP_3 receptor subtype specificity as well. However, as expected BUT did not attenuate AVP-dependent cAMP and no significant difference was observed for any concentration of BUT

incubated together with 10^{-7}M AVP compared to the cAMP levels observed for 10^{-7}M AVP alone (ANOVA).

While BUT elevates cAMP levels in our preparation of rat mTAL, suggesting the action of the EP_2 receptor subtype, this does not mean that the EP_4 receptor subtype has no role to play in the cAMP-generating response of PGE_2. Further experiments were done to differentiate PGE_2-dependent cAMP as EP_2 and/or EP_4 receptor subtype mediated. The cAMP response to 10^{-6}M PGE_2 and BUT was tested with and without the EP_4 receptor subtype antagonist AH-23848B (fig.3). No reversal of PGE_2 or BUT-mediated inhibition was observed in mTAL pretreated with 10^{-5}M AH-23848B, nor was there any significant difference observed between mTAL with and without antagonist pretreatment (ANOVA). These results suggest that the cAMP-generating effect of PGE_2 in the rat mTAL to be mediated through the EP_2 receptor subtype alone and that the EP_4 receptor subtype is not involved.

Micromolar concentrations of PGI_2 have been shown to increase cAMP levels in rat IMCD cells. No work has been done to investigate a potential PGI_2-dependent cAMP increase in rat mTAL. Therefore, studies were done looking at potential changes in cAMP levels mediated by the PGI_2 analogs ILP and CCP (fig. 4). No elevations were observed for any concentration of ILP and CCP tested. The dose-response range studied for the PGI_2 analogs was 10^{-11} to 10^{-6} M; no significant difference was observed between cAMP responses of the tested concentrations and those of the basal values (ANOVA). Based on this evidence, it appears that the PGI_2 analogs ILP and CCP alone do not generate cAMP in the rat mTAL.

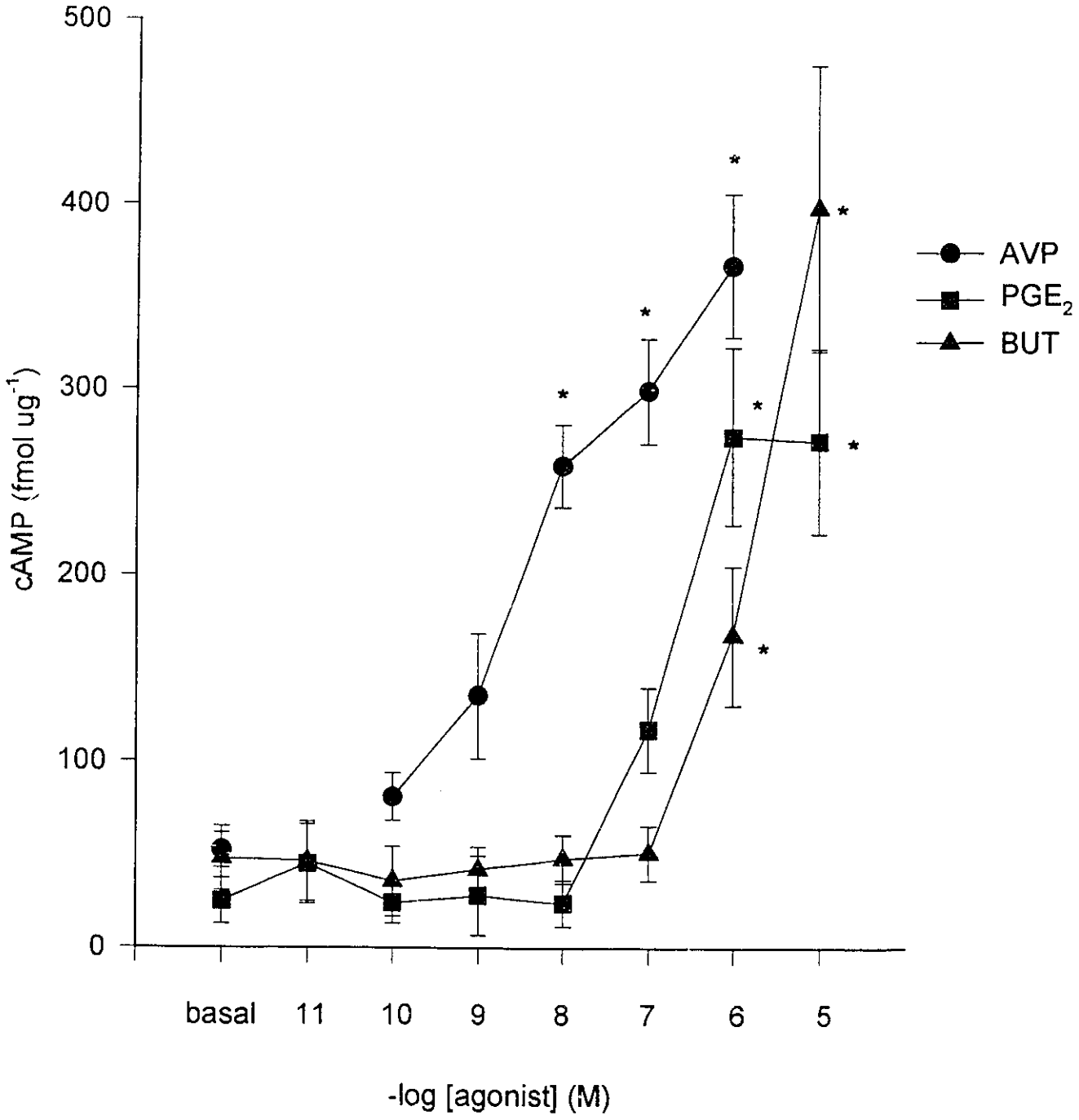

Fig. 1 Cyclic AMP stimulation in response to AVP, PGE_2 and Butaprost (BUT) in freshly isolated rat mTAL. Dose-response range tested for AVP was 10^{-10} to10^{-6}M, and 10^{-11} to 10^{-5}M for PGE_2 and BUT. n=4 $\pm$ SEM, * $p<0.05$ vs basal, ANOVA, Bonferroni t-test

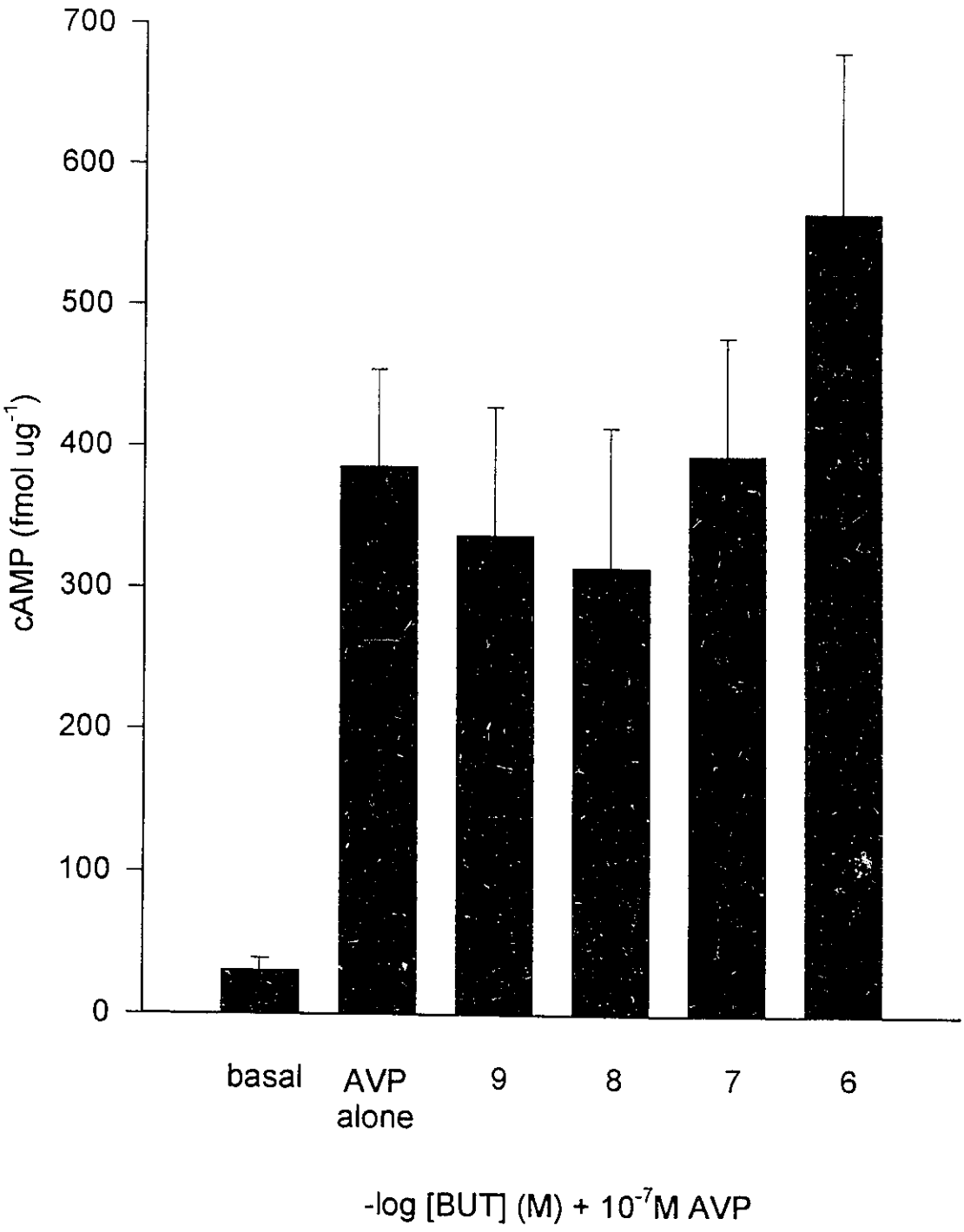

Fig. 2 AVP and BUT coincubation in rat mTAL. Cyclic AMP response was tested for AVP alone and AVP together with increasing doses of BUT from 10^{-9} to 10^{-6} M. n=4 $\pm$ SEM

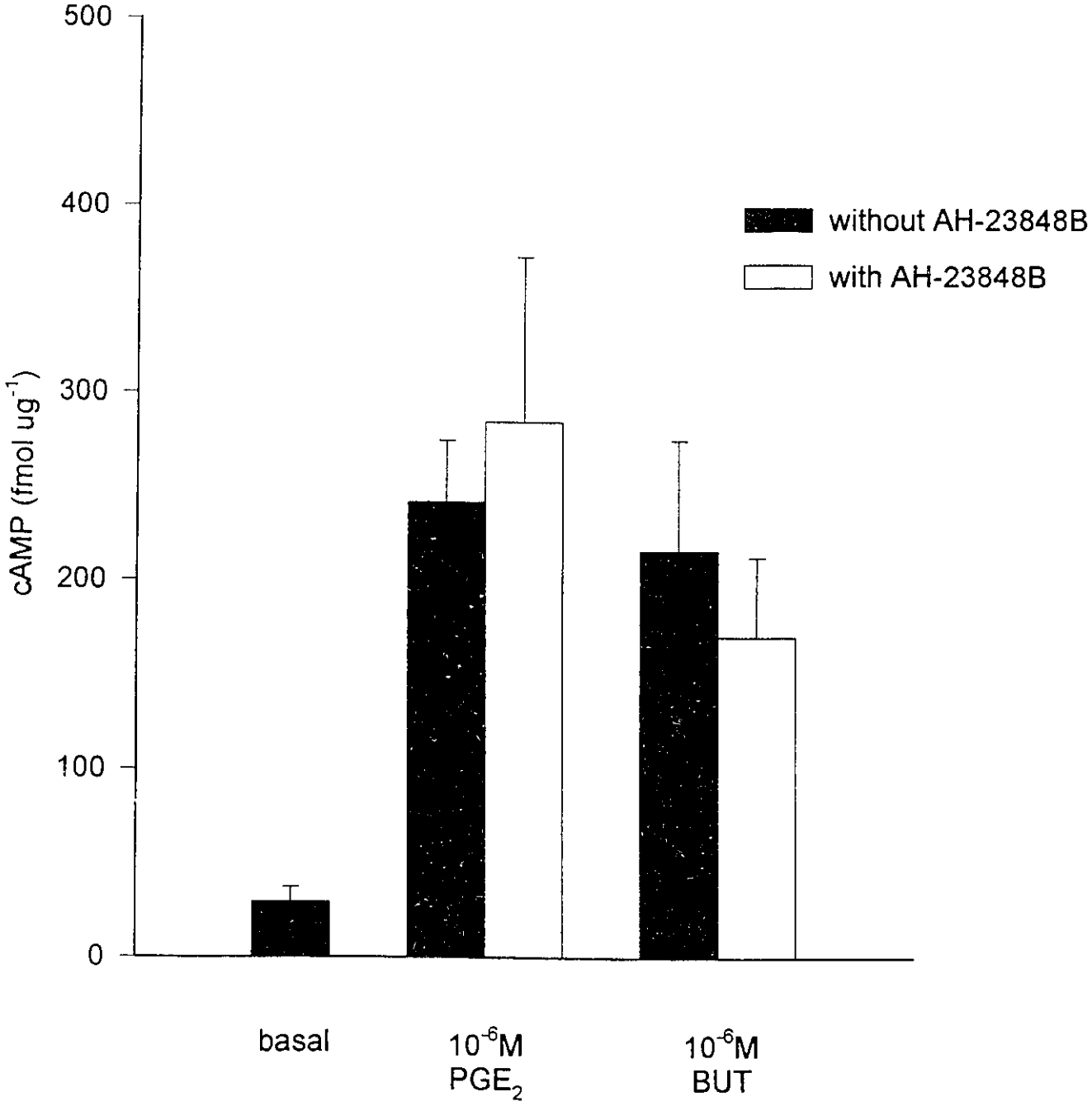

Fig. 3 PGE_2 and BUT-dependent cAMP pretreated with EP_4 receptor subtype antagonist AH-23848B. Rat mTAL were pretreated with 10^{-5} M AH23848B prior to incubation with PGE_2 and BUT. n=4 $\pm$ SEM

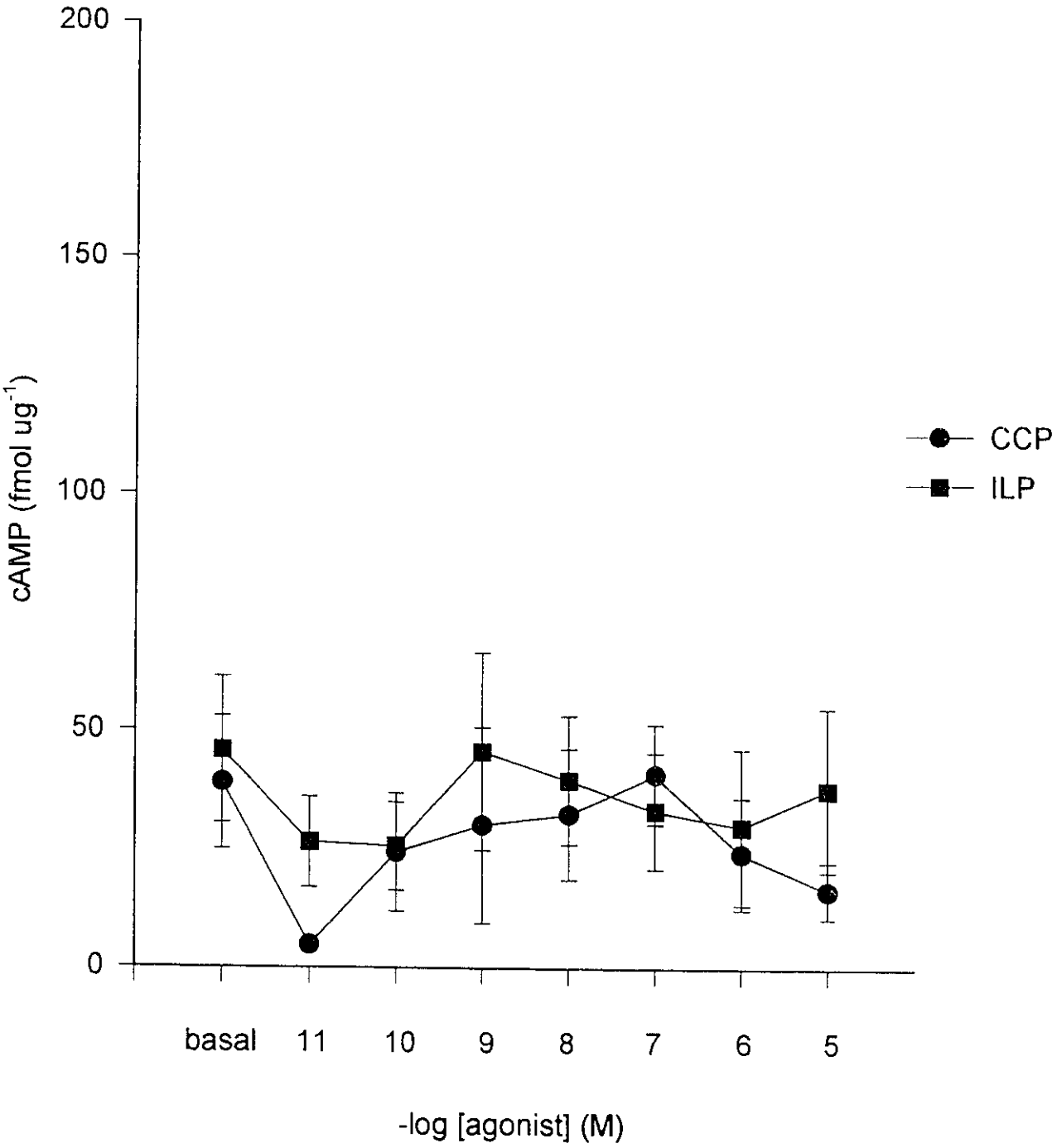

Fig. 4 Cyclic AMP accumulation in response to iloprost (ILP) and cicaprost (CCP) in freshly isolated rat mTAL. Dose-response range tested for both PGI_2 analogs was 10^{-11} to 10^{-5} M. n=4 $\pm$ SEM

3.1.2 Inhibition of AVP-dependent cAMP

It is known that PGE_2 will attenuate AVP-dependent cAMP in the rat mTAL (Torikai and Kurokawa 1983, Nakao et al. 1989, Firsov et al. 1994). Following the cAMP generating studies, it was decided to reproduce the PGE_2-mediated inhibition of AVP-dependent cAMP in our tissue preparation (fig.5). This was necessary before looking at any PGI_2 analog-dependent inhibition of AVP-dependent cAMP. Similar inhibitory actions on AVP-dependent cAMP were tested using the PGI_2 analogs ILP and CCP (fig. 6). Significant inhibition was achieved using PGE_2 at 10^{-9} and 10^{-8}M PGE_2 (ANOVA, Bonferroni t-test). Maximal inhibition was achieved at 10^{-8}M PGE_2 where AVP-dependent cAMP levels dropped 70.7% to 117.0 ± 33.1 fmol/ug. The effect of PGE_2 on AVP-dependent cAMP was biphasic. PGE_2 concentrations less than and equal to 10^{-8}M PGE_2 inhibited AVP-dependent cAMP, while cAMP levels began to increase at PGE_2 concentrations greater than 10^{-8}M. This evidence clearly shows that PGE_2 can inhibit AVP-dependent cAMP in our preparation of rat mTAL

The PGI_2 analogs ILP and CCP have both been shown to inhibit AVP-dependent cAMP in microperfused rabbit IMCD (Hébert et al. 1995). In this work, it was proposed that the PGI_2 analogs operate through a putative IP_3 receptor subtype which, like the EP_3 counterpart, is coupled to the inhibition of AVP-dependent cAMP through a Gi-regulatory protein. PGE_2 inhibits AVP-dependent functional effects in the mTAL as well. It may therefore be that PGI_2 compliments PGE_2-mediated inhibition of AVP-dependent cAMP in the rat mTAL. The dose-response inhibition of AVP-dependent cAMP was tested using ILP and CCP from 10^{-10}M to 10^{-}

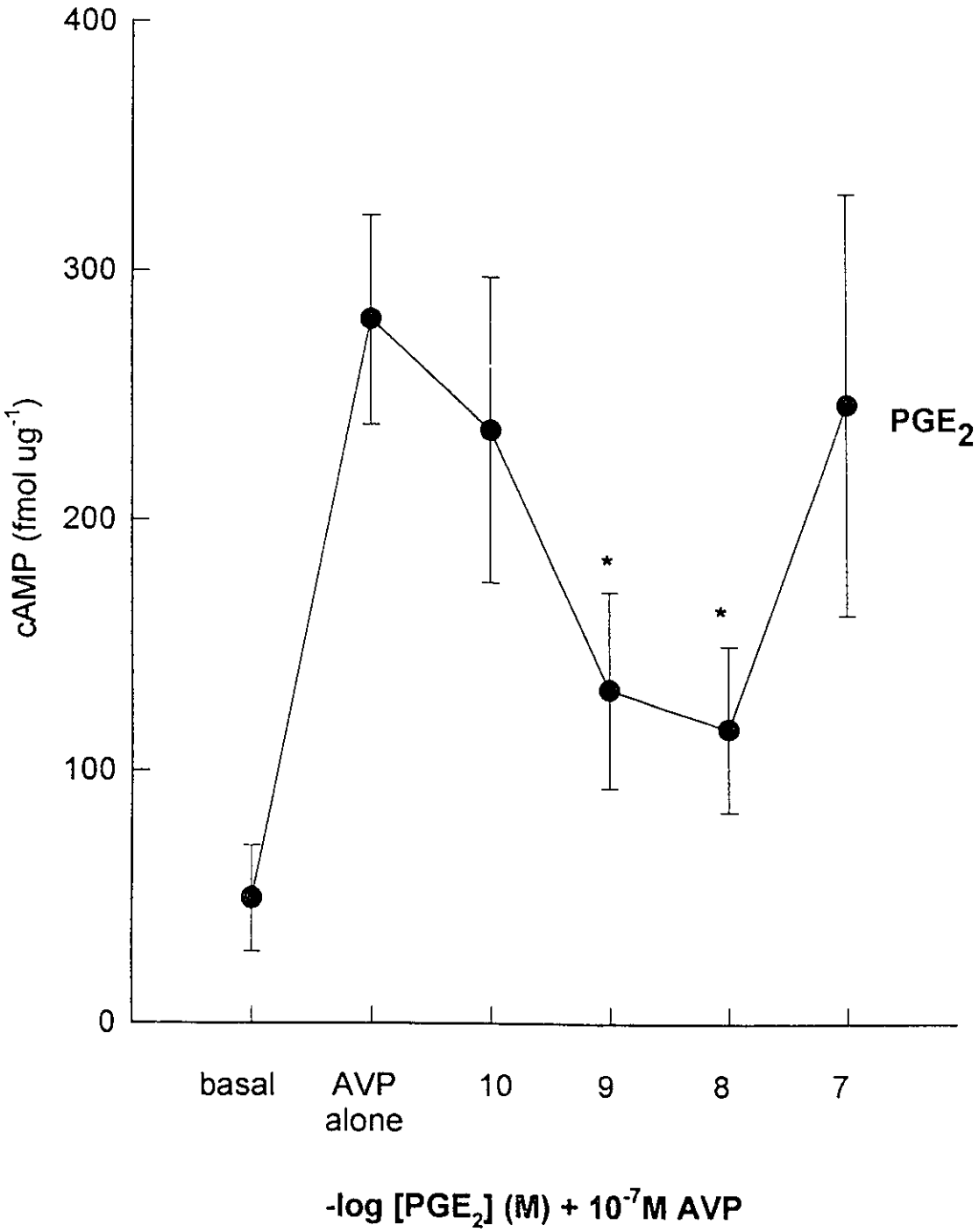

Fig. 5 PGE_2 dose-response inhibition of AVP-dependent cAMP. Cyclic AMP response was tested for AVP alone and together with increasing doses of PGE_2 from 10^{-10} to 10^{-7}M. n=5 $\pm$ SEM, * $p<0.05$ vs. AVP alone, AVOVA, Bonferroni t-test

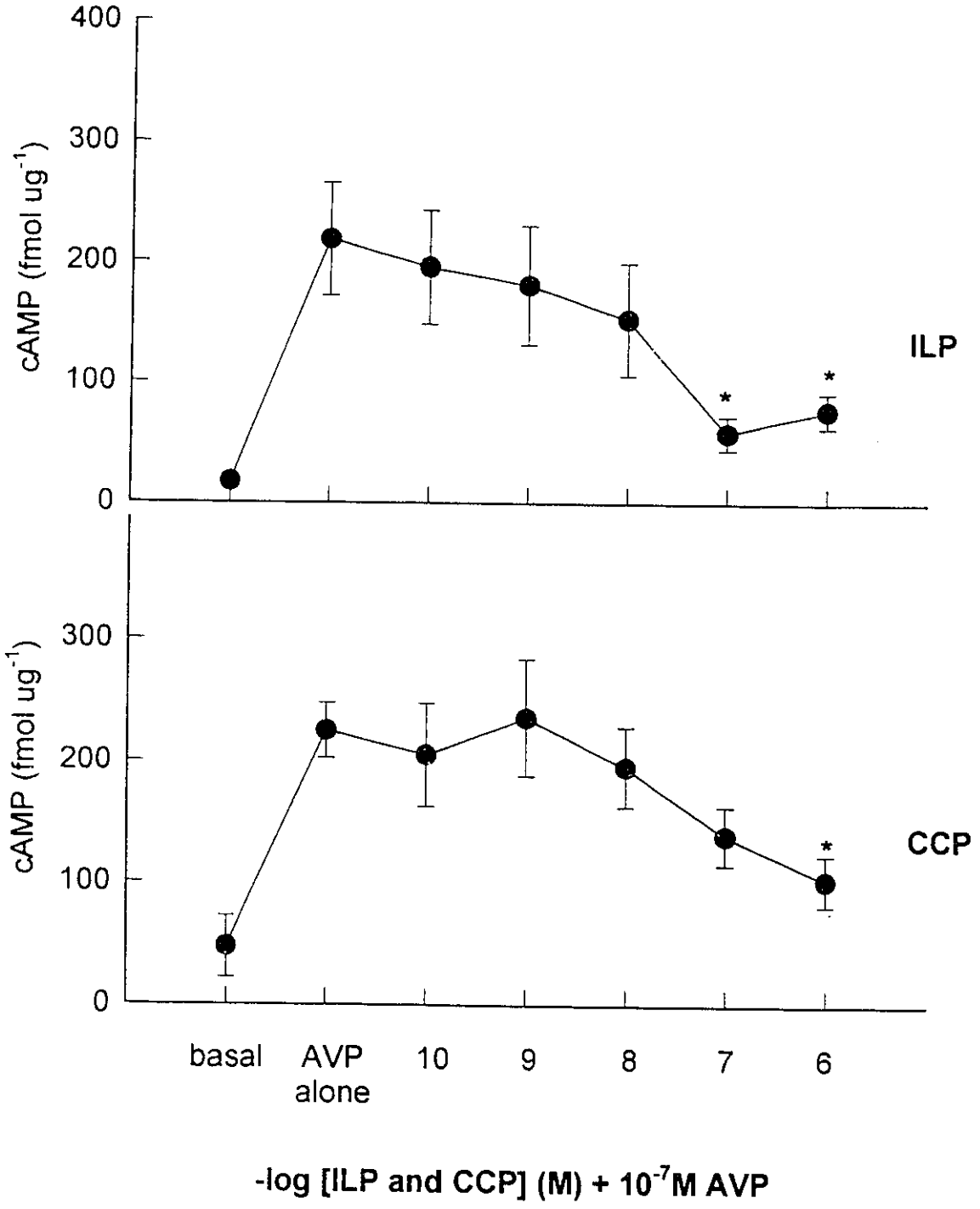

Fig. 6 ILP and CCP dose-response inhibition of AVP-dependent cAMP. Cyclic AMP response was tested to AVP alone and with increasing doses of PGI_2 analogue from 10^{-10} M to 10^{-6} M. n=5 $\pm$ SEM, * $p<0.05$, ANOVA, Bonferroni t-test

^{6}M (fig.6). Significant inhibition was achieved at 10^{-7} and 10^{-6}M ILP (ANOVA, Bonferroni t-test). Maximal inhibition for ILP was achieved at a concentration of 10^{-7}M where cAMP levels decreased 80% from a 10^{-7}M AVP-dependent maximum of 218 ± 46.8fmol/ug to 58.3 ± 13.8 fmol/ug. Significant and maximal inhibition was achieved with 10^{-6}M CCP, where cAMP levels dropped 68.9% above basal values from an AVP-dependent maximum of 225.1 ± 22.8 to 102.6 ± 20.7fmol/ug (ANOVA, Bonferroni t-test). This is the first evidence presented which shows that PGI_2 analogs, while alone appear to have no influence on cAMP levels, can inhibit AVP-dependent cAMP in the rat mTAL.

Our results at this point indicate that AVP-dependent cAMP is sensitive to PGE_2 and the PGI_2 analogs ILP and CCP. While this is suggestive of a potential role for an IP receptor in rat mTAL, the inhibitory effects observed using the PGI_2 analogs may also be mediated through the EP_3 receptor subtype. In an attempt to differentiate the EP_3 receptor subtype response from a potential IP receptor-mediated event, experiments were done examining for additive inhibition of AVP-dependent cAMP using PGE_2 and ILP (fig.7). Inhibitions of 68.9 and 76.7% were observed using 10^{-8} and 10^{-7}M PGE_2 and ILP respectively. These concentrations were chosen because they were the doses where maximal inhibition of AVP-dependent cAMP was observed in the dose response inhibition experiments. When the mTAL were preincubated with ILP first and then PGE_2, AVP-dependent cAMP was inhibited by 56.1%. However, no further inhibition was observed nor was there any significant difference between cAMP values from coincubation with PGE_2 and ILP compared to those from mTAL incubated with PGE_2 or ILP alone (ANOVA). There was no significant difference between the levels of inhibition achieved by PGE_2 and ILP alone compared to basal cAMP values (ANOVA, fig. 7).

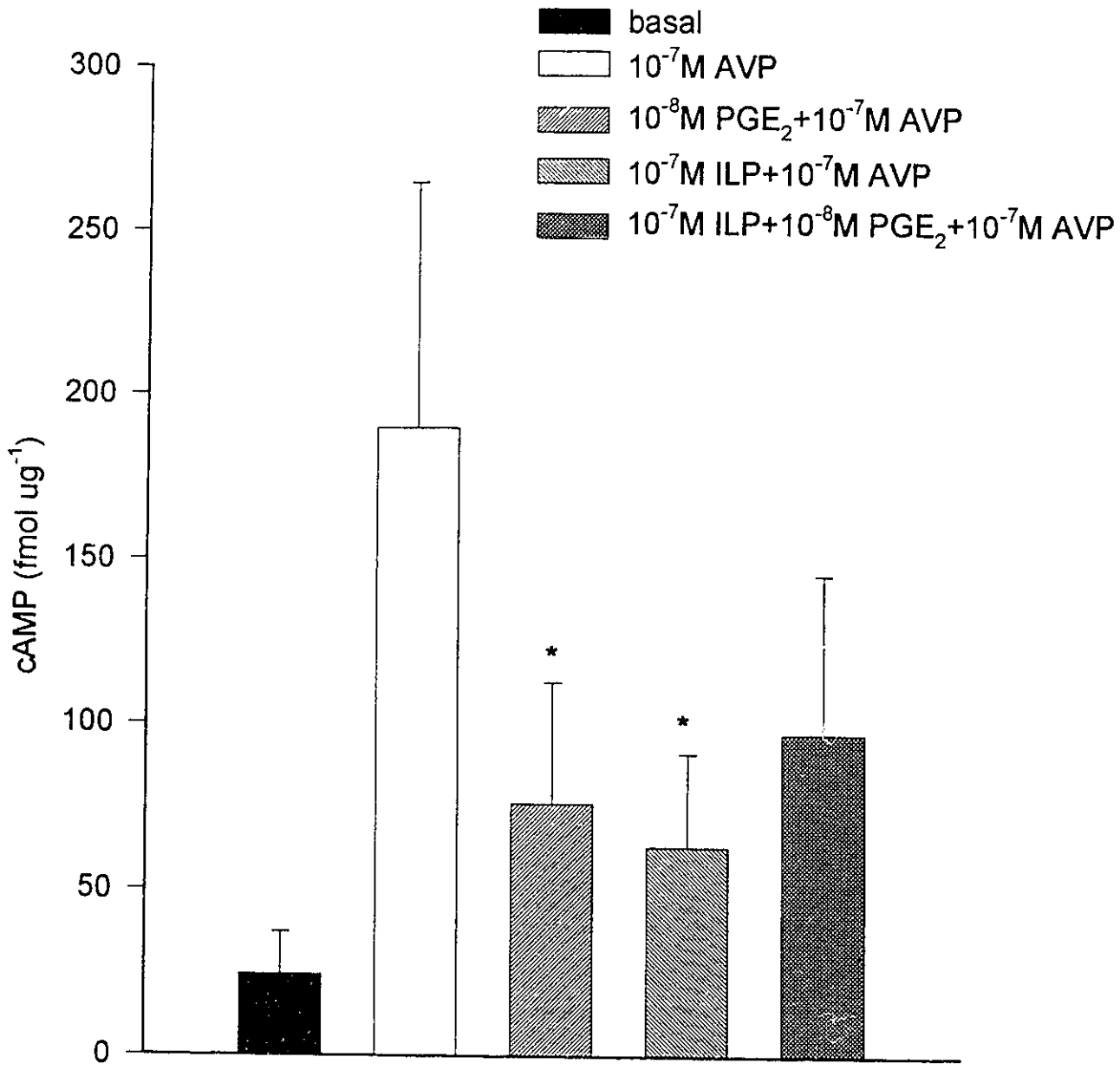

Fig. 7 Coincubation of rat mTAL with PGE_2 and ILP. Cyclic AMP response was tested with AVP alone; inhibition of AVP-dependent cAMP was tested with PGE_2 and ILP alone and together. n=4 $\pm$ SEM, * $p<0.05$ vs AVP alone, ANOVA, Bonferroni t-test

3.1.3 Mechanism of cAMP Modulation

3.1.3.1 Pertussis Toxin

Results from our experiments confirm previous studies which demonstrate inhibition of AVP-dependent cAMP with PGE_2. Other work has shown the mechanism of this inhibition to be coupled through a PTX-sensitive Gi-regulatory protein (Nakao et al. 1989, Firsov et al. 1994). Therefore, it was necessary to substantiate this fact and look at the possibility of a PTX-sensitive component underlying the inhibition of AVP-dependent cAMP mediated by ILP and CCP. A PTX sensitive component to the mechanism of action underlying PGE_2, ILP and CCP mediated inhibition of AVP-dependent cAMP was tested by pretreating the rat mTAL with 1ug/ ml PTX for 60 minutes (fig.8). PTX reversed the inhibition of AVP-dependent cAMP in the presence of 10^{-8}M PGE_2, 10^{-7}M ILP and 10^{-6}M CCP by 73.2, 72.5 and 73.3% respectively. The inhibition of AVP-dependent cAMP achieved by PGE_2, ILP and CCP was significantly different from mTAL incubated with AVP alone and in mTAL where the inhibition of AVP-dependent cAMP was reversed by PTX (ANOVA, Bonferroni t-test). With respect to actual values, the reversal of inhibition achieved by PTX was not complete. However, the cAMP levels induced by this reversal were not significantly different from those in mTAL incubated with AVP alone (ANOVA). Therefore, with respect to statistical significance, PTX completely reversed the inhibition of AVP-dependent cAMP mediated by PGE_2, ILP and CCP. No significant reversal

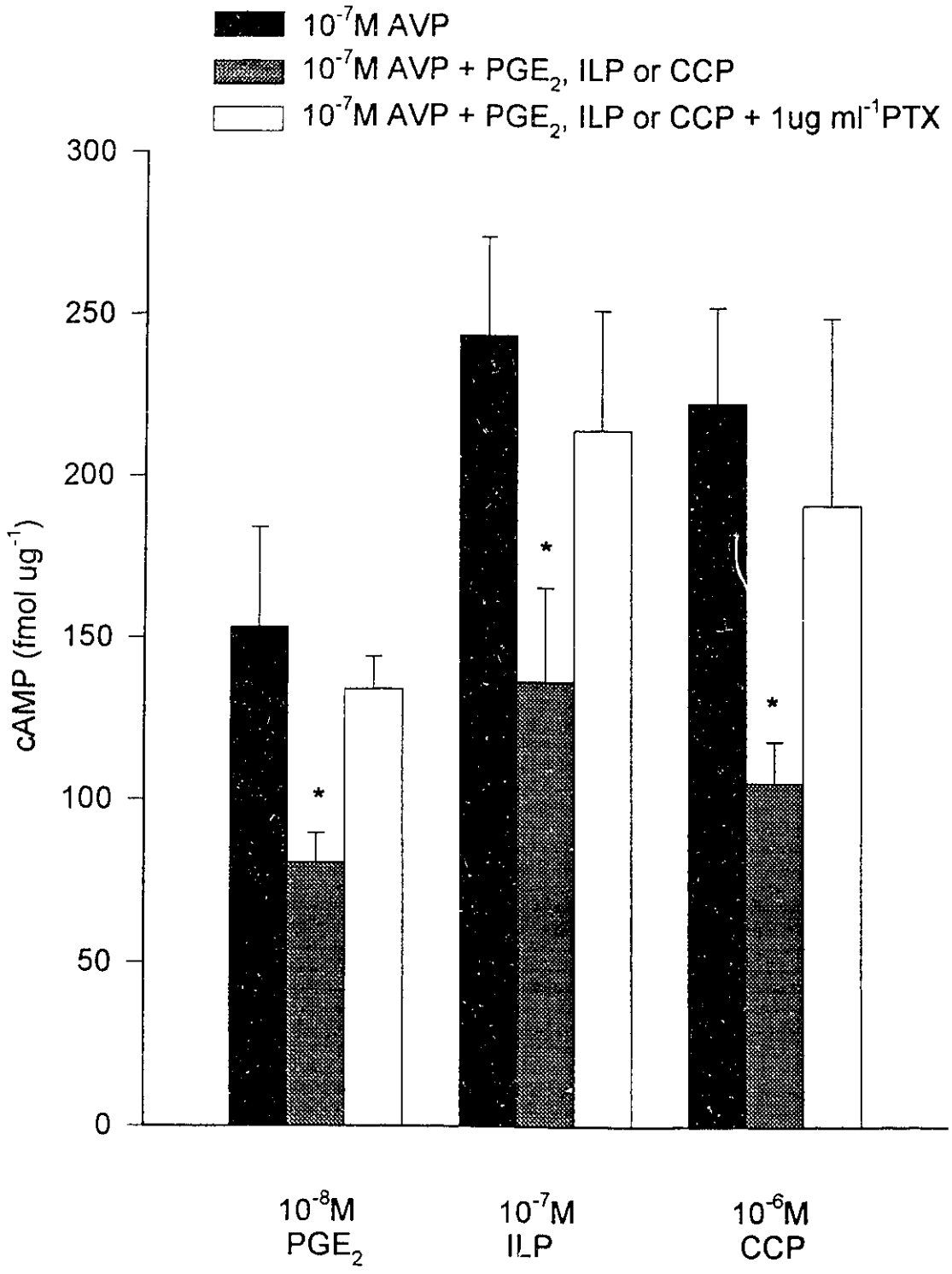

Fig. 8 Pertussis Toxin (PTX) reversal of PGE_2, ILP and CCP-mediated inhibition of AVP-dependent cAMP. n=5 $\pm$ SEM, * $p<0.05$ vs AVP alone and with PTX, ANOVA, Bonferroni t-test

was achieved by control groups where heat inactivated PTX (60°C for 2 hours) was substituted for biologically active PTX.

3.1.3.2 Protein Kinase C Inhibitors

cAMP values resulting from the reversal of inhibition of AVP-dependent cAMP by PGE_2, ILP and CCP in mTAL pretreated with PTX were not significantly different from mTAL incubated with AVP alone. This would indicate that PTX completely reverses the observed inhibition of AVP-dependent cAMP observed. However, evidence in rabbit CCD shows PGE_2-mediated inhibition of AVP-dependent cAMP to have both PTX and staurosporine, a PKC inhibitor, sensitive components (Noland et al. 1992). Based on the rationale that PGE_2 and the PGI_2 analogs may signal through similar mechanisms in the mTAL, a PKC-dependent component to the mechanism of action underlying the inhibition of AVP-dependent cAMP was also examined. Evidence from other studies indicates that cAMP levels are sensitive to PKC in some systems as a form of cell signaling cross-talk or feedback inhibition (Ando et al. 1988, Teitelbaum 1990, Kozawa et al. 1992). The prostanoid mediated inhibition of AVP-dependent cAMP was compared with and without pretreatment using PKC inhibitors bisindolylmaleimide (BIS.1) and calphostin C. PGE_2, ILP and CCP mediated inhibition were all insensitive to 30 minutes pretreatment with 2×10^{-6} M BIS.1 (fig.9). Also, no reversal of PGE_2 nor ILP mediated inhibition was observed in mTAL pretreated for 10 minutes with 10^{-7}M calphostin C (fig.10).

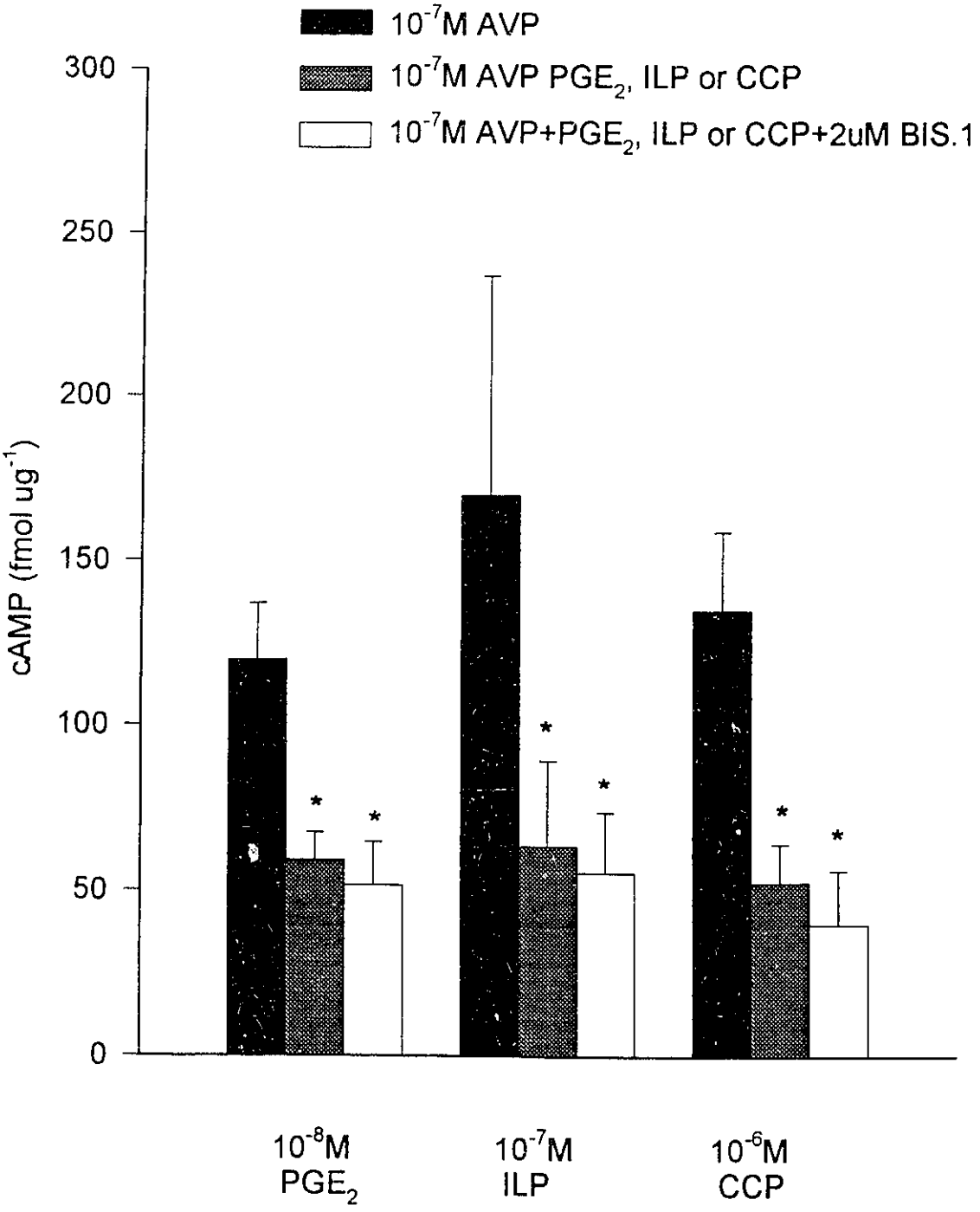

Fig. 9 Inhibition of AVP-dependent cAMP in rat mTAL pretreated with bisindolylmaleimide (BIS.1). Shown are responses to AVP and inhibition of AVP-dependent cAMP with and without BIS.1. n=4 $\pm$ SEM, * $p<0.05$ vs AVP alone, ANOVA, Bonferroni t-test

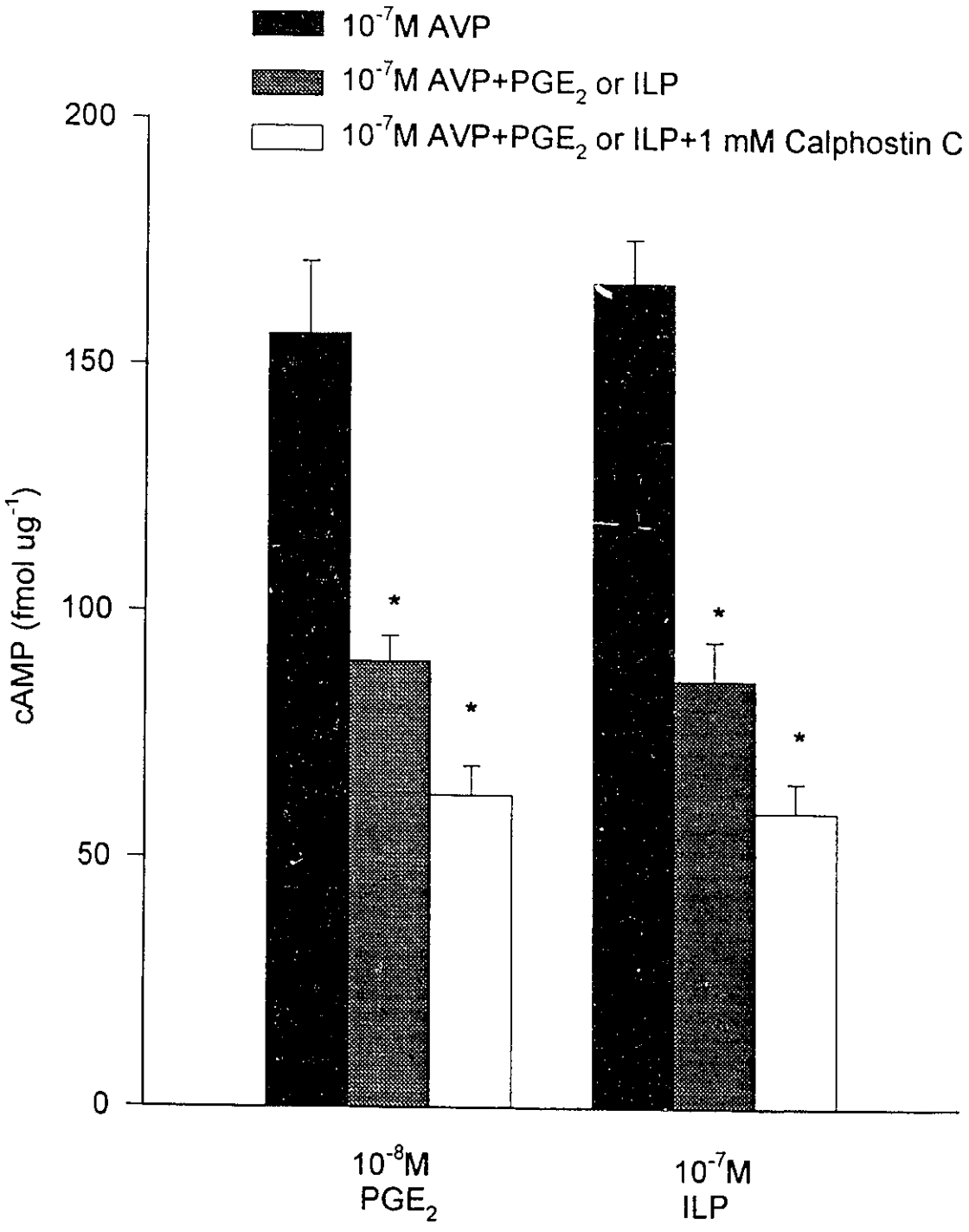

Fig. 10 Inhibition of AVP-dependent cAMP in rat mTAL pretreated with calphostin C. Shown are responses to AVP and Inhibition of AVP-dependent cAMP with and without Calphostin C pretreatment. n=4 $\pm$ SEM, * $p<0.05$ vs AVP alone, ANOVA, Bonferroni t-test

3.1.4 EP_1 Receptor Subtype Specific Antagonist AH-6809

The use of PKC inhibitors might characterize the presence of a receptor or receptor subtypes coupled to the hydrolysis of phosphatidyl inositol which might play a role in attenuation of AVP-dependent cAMP. Although no evidence was observed using PKC inhibitors, such a receptor may still be involved. PKC activation results from stimulation of PLC, which activates Ca^{2+} cell signaling. There is preliminary evidence which shows that arachidonic acid can raise intracellular Ca^{2+} in rat mTAL, suggesting the presence of an EP_1 receptor subtype (Firsov et al. 1994). We were further able to study the presence of the EP_1 receptor subtype which couples through this mechanism using the EP_1 receptor subtype specific antagonist AH-6809. Both PGE_2 and ILP mediated inhibition were compared in mTAL with and without 10^{-5}M AH-6809 pretreatment for 10 minutes (fig.11). No significant reversal of inhibition was observed (ANOVA); however, AH-6809 further attenuated PGE_2 mediated inhibition of AVP-dependent cAMP. The further attenuation of PGE_2-dependent inhibition of cAMP by AH-6809 pretreatment was not significantly different from those induced by ILP alone and ILP-dependent inhibition of cAMP with AH-6809 pretreatment. The inhibition achieved by PGE_2 alone may not represent a complete reversal in these experiments compared to what we had seen previously when we tested the PGE_2-dependent inhibition alone. ILP was also tested in these experiments because it has been shown that ILP exhibits weak agonist activity on the EP_1 receptor subtype. Therefore, the EP_1 receptor subtype does not appear to be involved in the PGE_2-mediated inhibition of AVP-dependent cAMP. These results compliment the evidence from the PKC-inhibition experiments which show no PKC-dependent component.

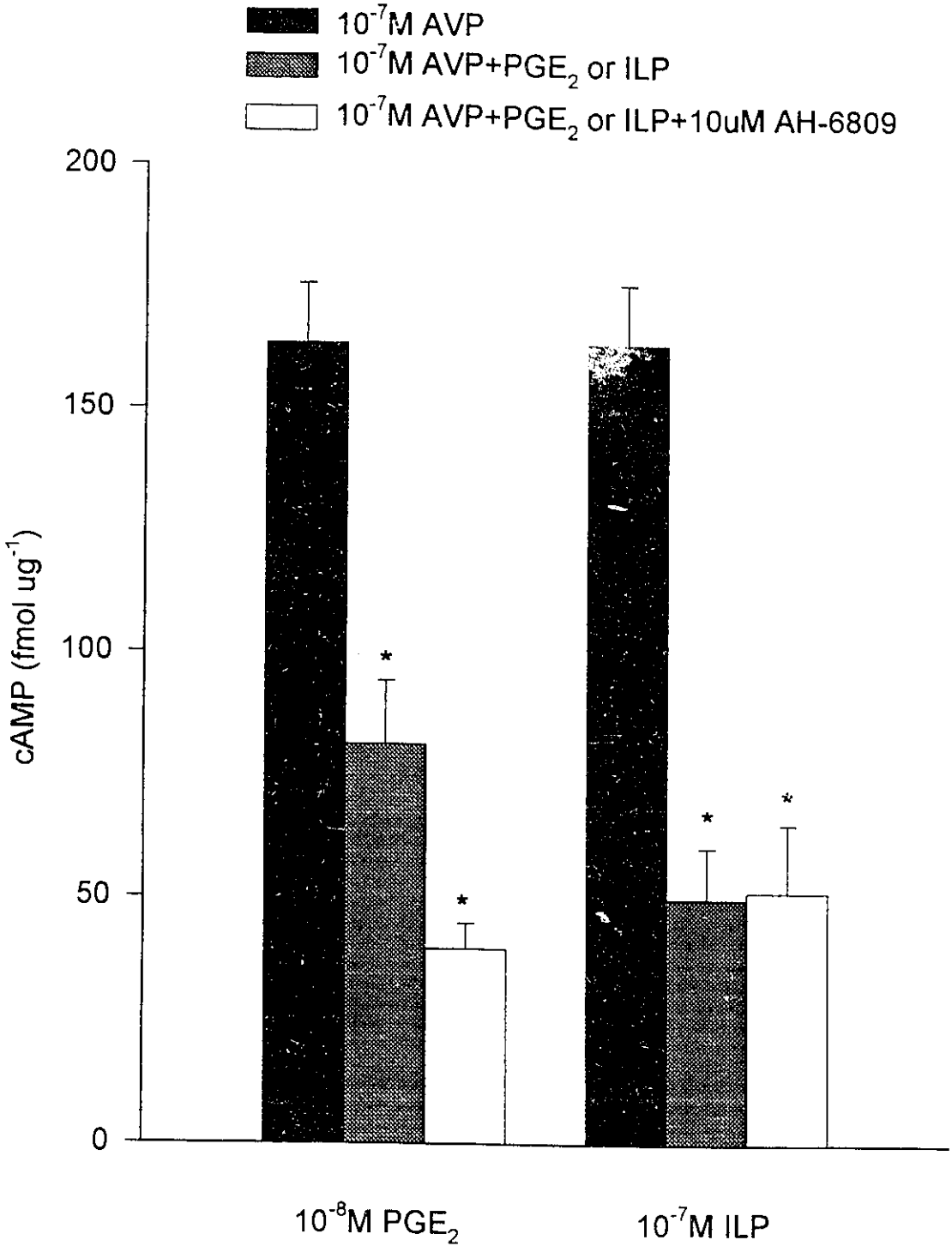

FIG. 11 Inhibition of AVP-dependent cAMP in rat mTAL pretreated with the EP_1 receptor subtype specific antagonist AH-6809. n=4 ± SEM,
* $p<0.05$ vs AVP alone, ANOVA, Bonferroni t-test

3.2 In Situ Hybridization

Five to ten percent contamination from outer medullary segments was present in the mTAL isolated in suspension for the cAMP studies. Non-mTAL segments, including the thin descending limb and the outer medullary collecting duct, may contribute to the cAMP responses observed. Therefore to support the cAMP data suggesting the presence of EP_2 and EP_3 receptor subtypes as well as the IP receptor in the mTAL region, in situ hybridization experiments were conducted to detect the expression of these receptors.

The initial molecular studies tried to colocalize in situ receptor expression with TH immunostaining on the same kidney section. We found that the Tamm-Horsfall immunostaining, although present, was substantially weaker on tissue sections where in situ hybridization was subsequently performed compared to sections which had been immunostained only. The results obtained on the photomicrographs were unconvincing in substantiating claims that a particular receptor could be colocalized to tubules that stained Tamm-Horsefall positive as well.

While future work will involve further attempts to achieve successful colocalization on the in same tissue section, for the purposes of this thesis receptor expression was localized to the mTAL by associating in situ receptor expression and TH immunostaining on similar mTAL patterns from adjacent sections of rat kidney tissue. This involved immunostaining for Tamm-Horsefall glycoprotein on one section and in situ hybridization on the adjacent section.

3.2.1 The EP_3 Receptor Subtype

The results of cAMP experiments provide evidence for the existence of the EP_3 receptor subtype in the rat mTAL. PGE_2 was shown to inhibit AVP-dependent cAMP through a pertussis toxin sensitive mechanism. This work confirmed previous work demonstrating the presence of the EP_3 receptor subtype in the mTAL (Breyer et al. 1996, Sugimoto et al. 1994, Breyer et al, 1993).

Intense EP_3 labeling to the outer medulla was seen in both 100x dark field photomicrographs (fig.12) and X-ray autoradiography (fig.13). Comparison of the Tamm-Horsefall immunostained to the 100x bright field photomicrographs sections from the adjacent tissue section shows a similar pattern of tubule formation (fig.14). Further detailed analysis of 400x bright field photomicrographs of the Tamm-Horsefall immunostained (fig.15) and in situ hybridized (fig.16) djacent sections clearly associates the expression of the EP_3 receptor subtype with the mTAL. Extreme care was taken in locating the same tubule pattern and actual tubules on the adjacent tissue sections. This was done through visual analysis and making sure the similar tubule patterns observed on adjacent tissue sections were equidistant with reference to nearby fiduciary marks. Probe specificity is established by comparison of the labeling of the antisense oligo (fig.12) to the absence of labeling of the control sense stranded oligo (fig.17). Probe specificity is also demonstrated in the X-ray autoradiography shown in fig.13. As well, Tamm-Horsefall immunostaining is clearly specific as indicated by the absence of DAB stain on the control sections where the primary goat antiserum was substituted with non-immune serum from goat. A 400x bright field photomicrograph of TH control immunostaining of the adjacent section to TH-positive immunostaining (fig.16) is shown in fig.18.

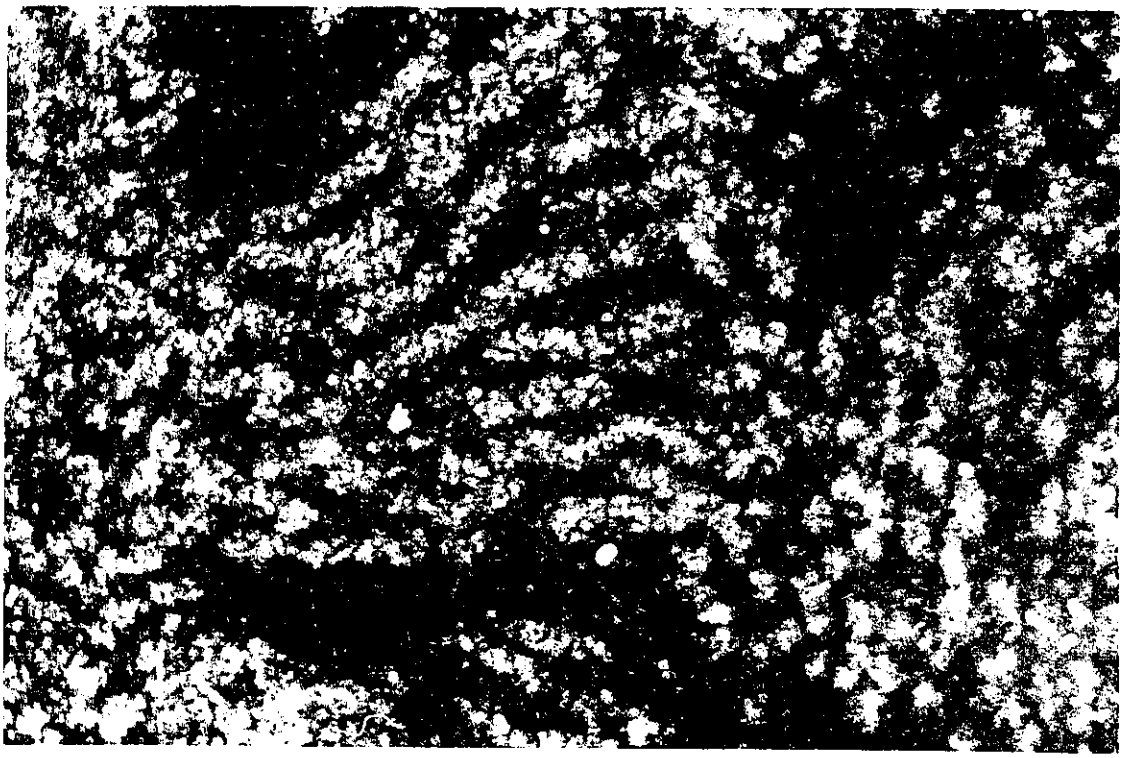

Fig. 12 100x dark field photomicrograph demonstrating positive labelling of ^{35}S-labelled anti-sense oligo probe for the EP_3 receptor subtype in the outer medullary region. Exposure to photographic emulsion gel was for seven days.

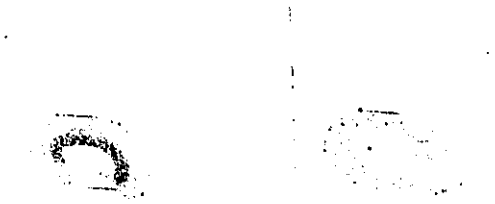

Fig.13 X-ray autoradiography demonstrating positive labelling for ^{35}S-labelled EP_3 receptor subtype oligo probe. Anti-sense oligo is shown on the left and the sense-strand oligo is shown on the right.

Fig. 14 100x bright field photomicrograph demonstrating positive immunoperoxidase staining for Tamm-Horsfall glycoprotein on the TAL in the outer medullary region. This tissue section is adjacent to that shown in fig. 12.

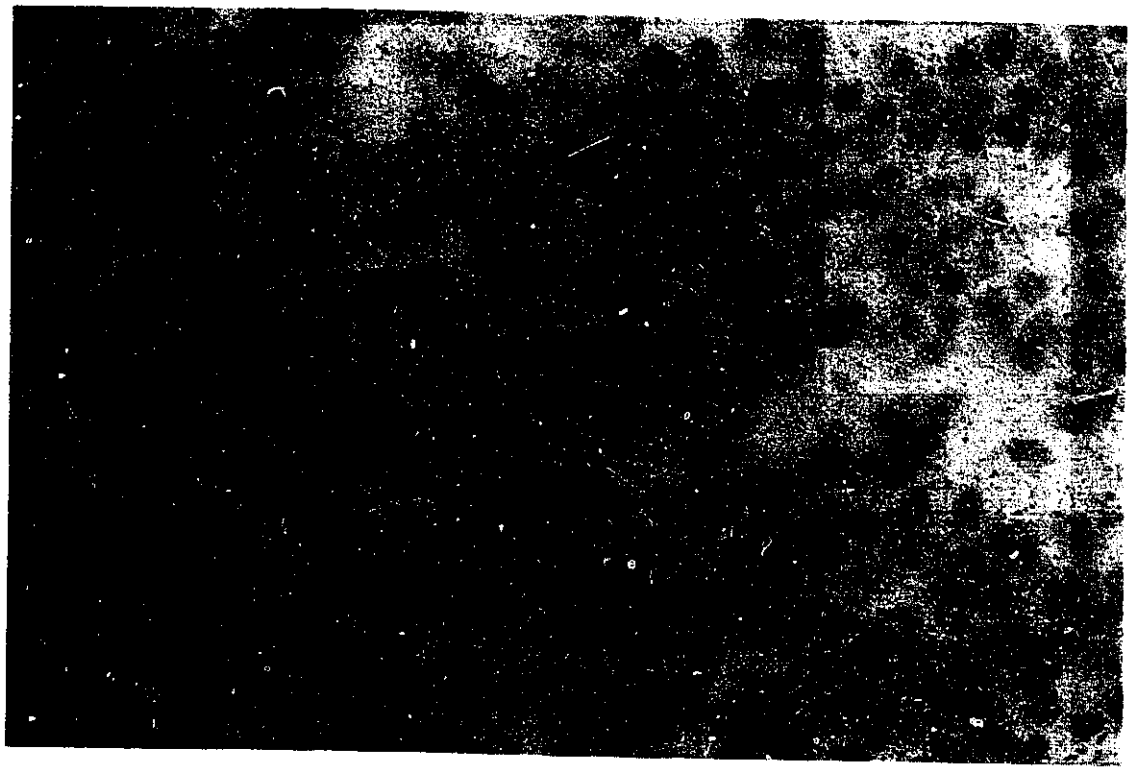

Fig. 15 400x bright field photomicrograph demonstrating positive labelling for ^{35}S-labelled anti-sense oligo probe for the EP_3 receptor subtype in the outer medulla.

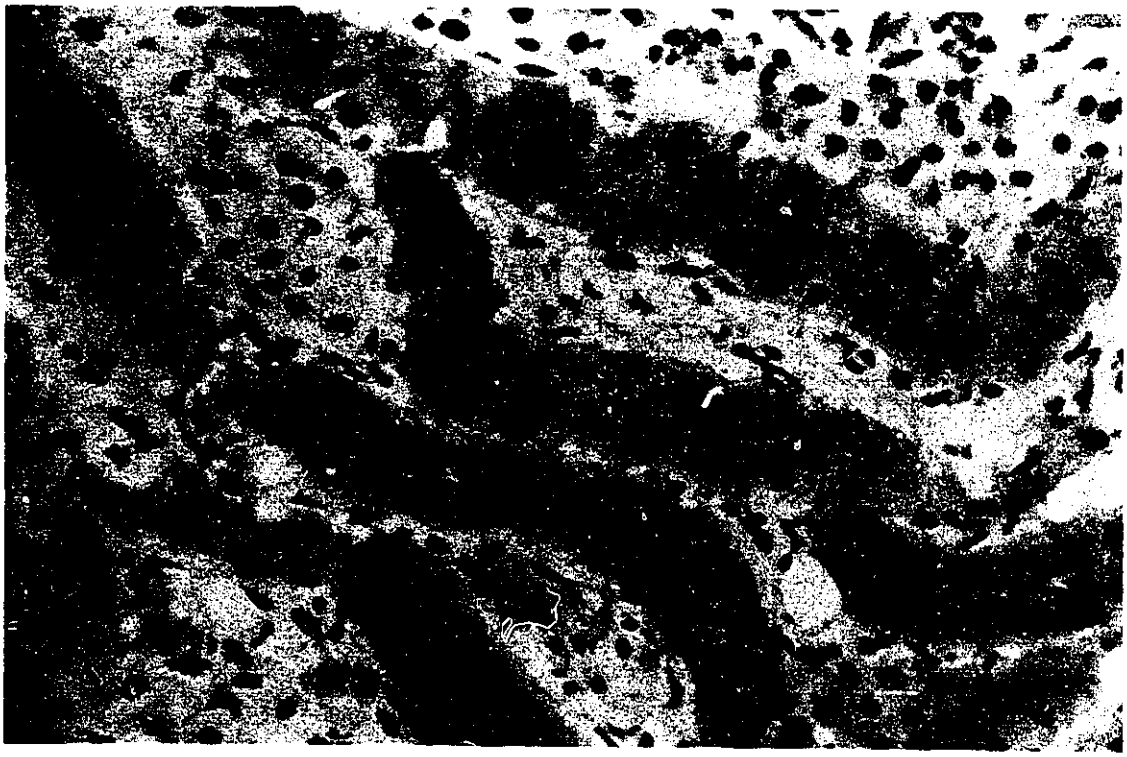

Fig.16 400x bright field photomicrograph demonstrating positive immunoperoxidase staining for Tamm-Horsfall glycoprotein on the TAL. This tissue section is adjacent to that shown in fig. 15.

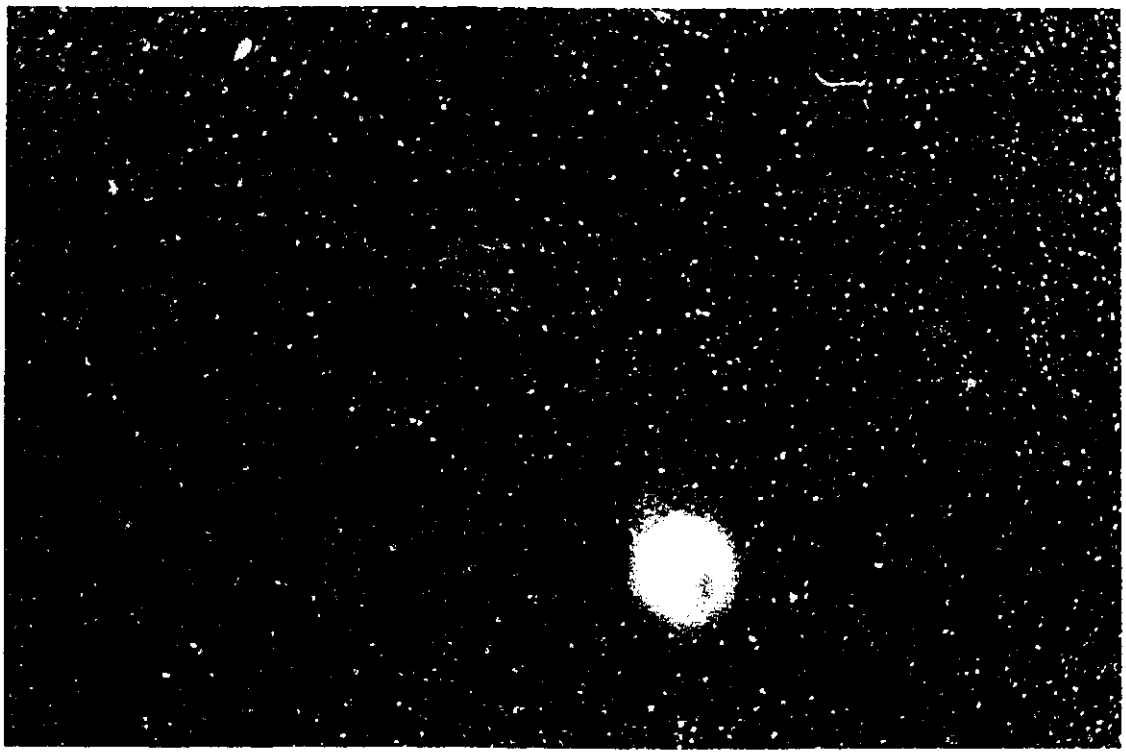

Fig. 17 100x dark field photomicrograph demonstrating negative labelling of ^{35}S-labelled sense strand oligo probe for the EP_3 receptor subtype in the outer medulla.

Fig. 18 400x bright field photomicrograph demonstrating negative immunoperoxidase staining for Tamm-Horsfall glycoprotein in the TAL.

3.2.2 The EP_2 Receptor Subtype

The biochemical evidence presented earlier suggests the presence of the EP_2 receptor subtype in the rat mTAL. Both PGE_2 and the EP_2 receptor subtype agonist butaprost increased cAMP levels in the mTAL suspension. Attempts to inhibit the cAMP response to PGE_2 by pretreatment with the EP_4 receptor subtype antagonist AH-23848B (Glaxo-Wellcome) were unsuccessful. This suggests that the EP_2 receptor subtype is responsible for the PGE_2-dependent cAMP response observed in the rat mTAL and that the EP_4 receptor subtype is not involved.

To further substantiate the evidence that the EP_2 receptor subtype is present in the rat mTAL, we conducted in situ hybridization studies looking for the expression of this receptor subtype. Following hybridization, the sections were exposed to X-ray film for four days and photographic emulsion gel up to four weeks. Neither X-ray autoradiography nor 100x dark field photomicrographs demonstrate the presence of the EP_2 receptor subtype mRNA in the outer medulla of the rat kidney. There was no detectable visible difference observed in 100x dark field photomicrographs between sections incubated with antisense (fig.19) and sense stranded (fig.20) oligo probes. Analysis of 400x bright field photomicrographs provided no clear evidence of the EP_2 receptor subtype mRNA in any tubule structures in the outer medulla. Therefore, the in situ hybridization studies do not corroborate the cAMP evidence in substantiating the presence of the EP_2 receptor subtype in the rat mTAL. No labeling was present in any region of the kidney for EP_2 receptor in situ hybridization.

Fig. 19 100x dark field photomicrograph demonstrating negative labelling of ^{35}S-labelled anti-sense oligo probe for the EP_2 receptor subtype in the outer medullary region. Exposure to photographic emulsion gel was for four weeks.

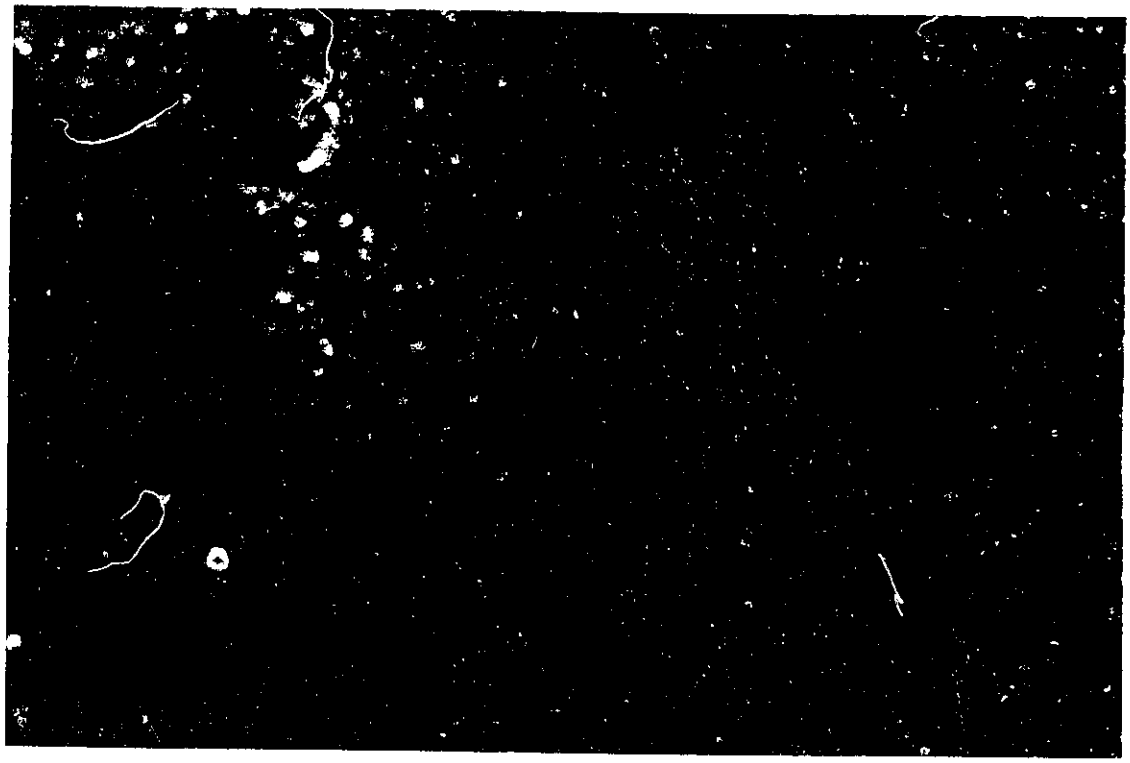

Fig. 20 100x dark field photomicrograph demonstrating negative labelling of ^{35}S-labelled sense-strand oligo probe for the EP_2 receptor subtype in the outer medulla.

3.2.3 The IP Receptor

Results from our cAMP experiments suggest that the rat mTAL has the prostacyclin receptor. Using the PGI_2 analogs iloprost and cicaprost, inhibition of AVP-dependent cAMP was observed. Also, this is dependent on a Gi regulatory protein-coupled mechanism because the inhibition of AVP-dependent cAMP was sensitive to pertussis toxin. There were concerns however, that the prostacyclin-dependent inhibition was the result of an event occurring at the EP_3 receptor subtype. Coincubation of PGE_2 and iloprost produced no additivity of inhibition of AVP-dependent cAMP which, combined with the observation that both PGE_2 and ILP/CCP mediated inhibition are sensitive to pertussis toxin, validated our concern that the prostacyclin analogs were operating through the EP_3 receptor subtype. Therefore further characterization of the IP receptor through in situ hybridization experiments took on added significance. First, as with the E-prostanoid receptor subtypes studied, it was necessary to associate expression of the IP receptor with Tamm-Horsfall immunostaining of the rat mTAL. Secondly, and just as important, successful in situ hybridization demonstrating the expression of the IP receptor suggests that the PGI_2 analog-dependent cell signaling responses are independent of the EP_3 receptor subtype.

Dark field 100x photomicrographs show positive labeling using the antisense oligo probe in the outer medulla of rat kidney sections (fig.21). The positive labeling observed with the IP oligo probe is not as strong as was observed for the EP_3 oligo probe; however, there is still substantial labeling compared to the background levels demonstrated on the control sections where the sense stranded control oligo was used (fig.22). Comparison of similar outer medullary

tubule patterns from the 100x dark field photomicrographs showing in situ IP labeling and immunostaining for Tamm-Horsefall on serial sections (fig.23) suggests that the IP receptor subtype is expressed in the rat mTAL. Exposure times required to obtain this level of in situ labeling for the IP receptor mRNA were longer than they were for the EP_3 receptor subtype experiments. After dipping the tissue sections into the photographic emulsion gel, they were exposed and developed at one, two, three, and four weeks. It was found that an exposure time of three weeks was required to attain the level of labeling observed in the 100 x dark field photomicrographs. Unfortunately at the time of writing, we do not have X-ray autoradiographs demonstrating the results we see in the photomicrographs; however, these experiments are underway. Tissue sections were exposed to X-ray film for four days, but no clear labeling was observed. Also, detection and photomicroscopy of the IP labeling was only possible under dark field conditions. At the low level of IP receptor expression, bright field photomicrographs show no clear labeling of IP receptor mRNA in the tissue sections. Therefore, IP receptor expression is associated with the rat mTAL; however, levels of expression appear lower than for those observed for expression of the EP_3 receptor subtype.

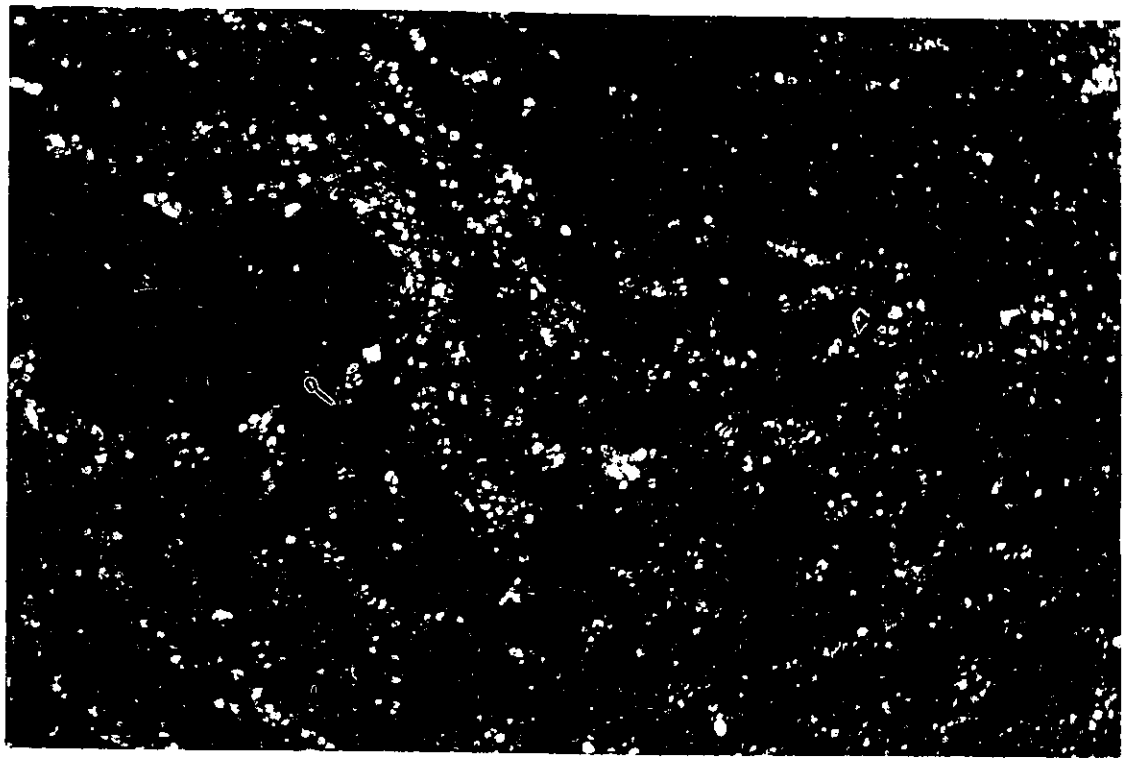

Fig. 21 100x dark field photomicrograph demonstrating positive labelling for ^{35}S-labelled anti-sense oligo probe for the IP receptor in the outer medulla. Exposure to photographic emulsion gel was for three weeks.

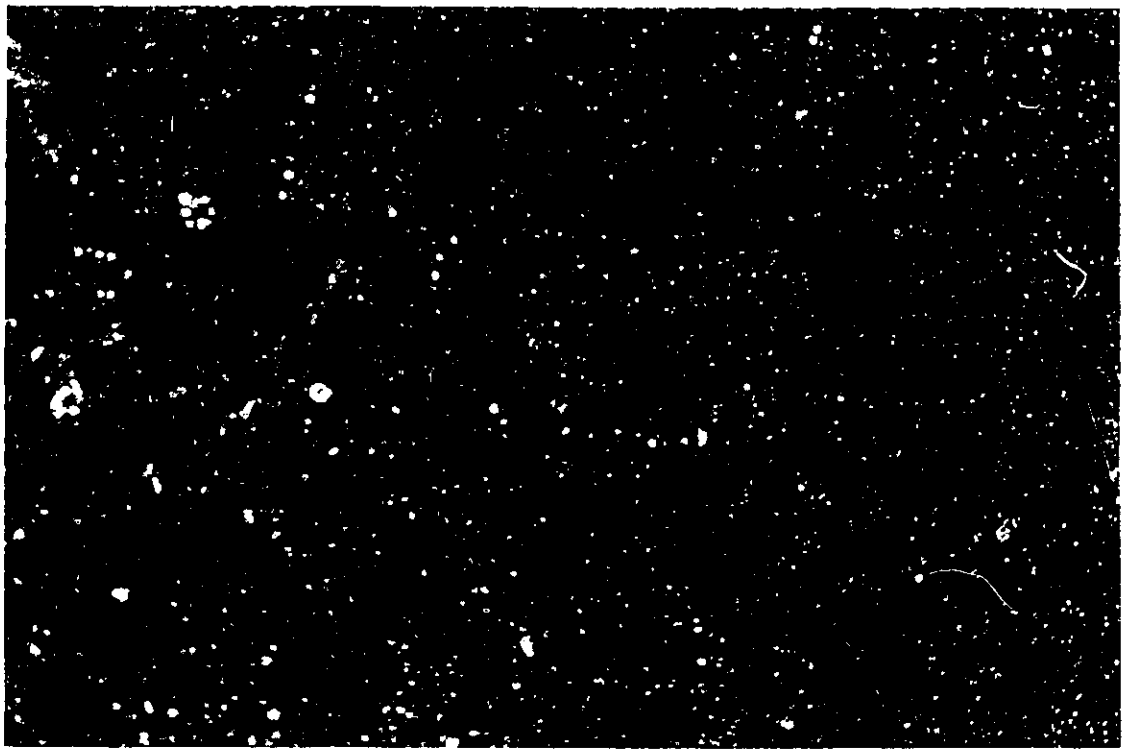

Fig. 22 100x dark field photomicrograph demonstrating negative labelling for ^{35}S-labelled sense-strand oligo probe for the IP receptor in the outer medulla.

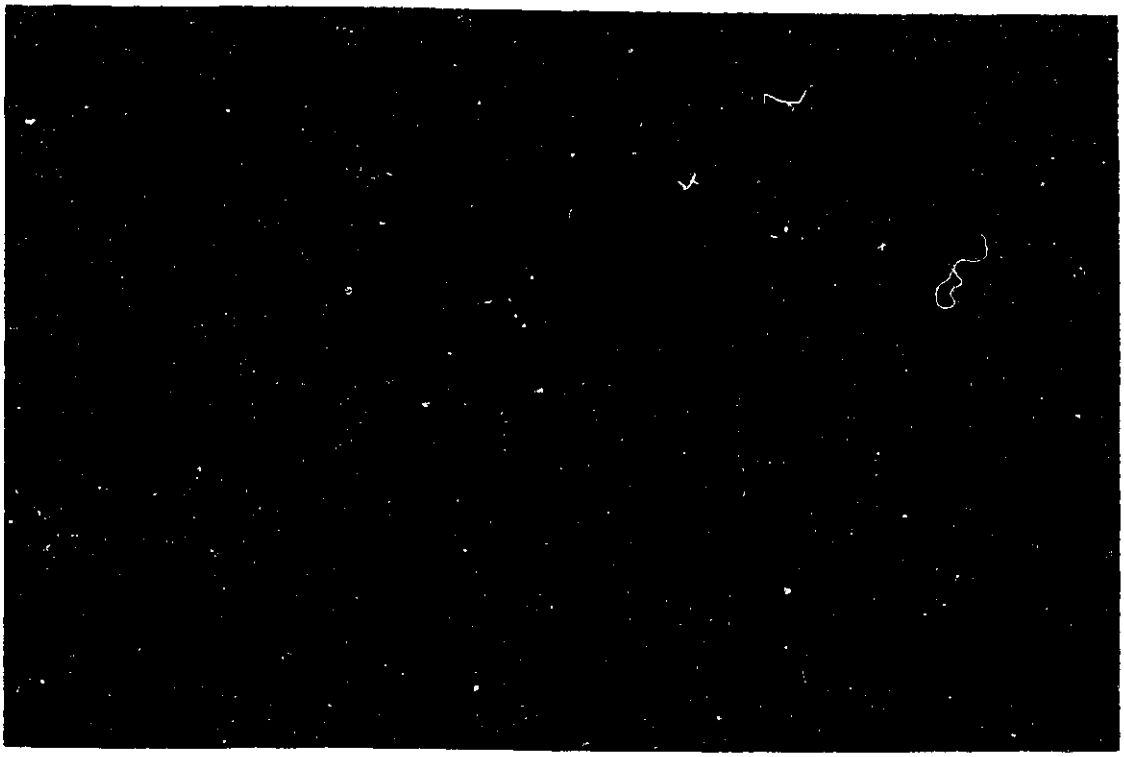

Fig. 23 100x bright field photomicrograph demonstrating positive immunoperoxidase staining of Tamm-Horsfall glycoprotein in the TAL. The tissue section shown in this figure is adjacent to that shown in fig. 21.

Section 4: Discussion

4.0 Discussion

4.1 The EP_3 Receptor Subtype

Inhibition of AVP-dependent-cAMP was observed in our preparation of rat mTAL using nanomolar concentrations of PGE_2. In our experiments, significant inhibition of AVP-dependent cAMP was achieved using 10^{-8} and 10^{-9}M PGE_2. This confirms previous work where PGE_2 has been shown to attenuate AVP-dependent cAMP in immunodissected cells and microdissected nephron segments from rat mTAL (Torikai and Kurokawa 1983, Nakao et al. 1989, Firsov et al. 1994). Upon further examination of the PGE_2 dose-response inhibition experiments, we noticed a biphasic effect. Inhibition of AVP-dependent cAMP increased proportionately with PGE_2 concentrations from 10^{-11} to 10^{-8}M, where maximal inhibition was achieved. However, with successive increases in PGE_2 concentration from 10^{-8} to 10^{-6}M, cAMP levels increased from the point of maximal inhibition. Also, in experiments where the mTAL suspension was incubated with PGE_2 alone, only a cAMP-generating response was observed.

There appears to be two different populations of E-prostanoid receptors in the rat mTAL; one is coupled to the generation of cAMP, the other to inhibition of AVP-dependent cAMP. This biphasic response provided some clues as to why when the mTAL suspension is incubated with PGE_2 alone a cAMP-generating response is observed and when pretreated with PGE_2 followed by AVP incubation, inhibition of AVP-dependent cAMP is observed. The receptor subtype coupled to the inhibition of AVP-dependent cAMP may represent a greater proportion of these PGE_2 binding sites and/or may have a higher affinity for PGE_2. In such a case, PGE_2 would

preferentially bind the inhibitory receptor subtype first. After these binding sites have become occupied, the PGE_2 may then bind to the receptor subtypes coupled to the generation of cAMP. Such a sequence of events would result in inhibition of previously elevated cAMP levels at lower doses of PGE_2 followed by an increase in cAMP at higher concentrations of PGE_2 where the inhibitory binding sites are maximally occupied and the stimulatory ones are being increasingly occupied and activated.

If two different E-prostanoid receptor subtype populations exist in the rat mTAL, why is inhibition of cAMP not observed when the mTAL suspension is incubated alone at the lower concentrations of PGE_2? It may be that the basal cAMP levels observed for controls may represent an absolute minimum level. The action of the inhibitory binding sites may be observed only when cAMP levels have been previously elevated. In both experiments where the mTAL suspension was incubated with PGE_2 alone or with both PGE_2 and AVP, increases in cAMP were observed only at 10^{-7} and 10^{-6}M PGE_2. This suggests that it is the stimulatory binding site might be responsible for the reversal of inhibition on AVP-dependent cAMP observed at the higher PGE_2 concentrations. There is evidence which suggests that the inhibitory receptor subtype represents a majority of the PGE_2 binding sites along the mTAL (Watanabe and Smith 1986, Breyer et al. 1993). While our evidence indicates the presence of two E-prostanoid receptor subtype populations, a definitive experiment to compare the relative proportions of these two receptor subtypes would be to quantify labeling of ^{3}H-PGE_2 and radiolabelled agonists such as the EP_2 receptor subtype-specific butaprost and antagonists such as the EP_3 receptor subtype-specific enprostil.

The inhibition of AVP-dependent cAMP observed using PGE_2 was a result of attenuated synthesis. Because the mTAL suspension in all experiments where cAMP was measured was pretreated with Ro20-1724, we believe the net inhibition of cAMP observed to be the result of a decreased rate of synthesis. Phosphodiesterase (PDE) is the class of enzyme responsible for the breakdown of cAMP to 5'-AMP; Ro20-1724 is a specific inhibitor of PDE and is more potent than the traditionally used isobutyl methylxanthene (IBMX). Making clear the relative roles of cAMP synthesis and degradation is a valid concern because Torikai and Kurokawa (1983) had shown that PGE_2-mediated inhibition of AVP-dependent cAMP to be a result of increased cAMP degradation in the cortical collecting tubule of the rat kidney. In our suspension of rat mTAL, PGE_2-mediated inhibition of AVP-dependent cAMP was the result of a reduced rate of cAMP synthesis.

The mechanism of action underlying the PGE_2-mediated inhibition of AVP-dependent cAMP is through Gi-regulatory protein coupling of the inhibitory receptor subtype to AC. Further experiments we conducted highlight the sensitivity of the PGE_2-induced inhibition of AVP-dependent cAMP to pertussis toxin (PTX). We were able to achieve 73% reversal of PGE_2-mediated inhibition of AVP-dependent cAMP by pretreating the freshly isolated suspension of rat mTAL with 1ug/ml PTX for 60 minutes at 37°C. This reversal is complete as the differences in cAMP levels observed between mTAL incubated with AVP alone and the PTX-pretreated group are not statistically significant. The results of these experiments using PTX confirm the previous work by Nakao et al. (1989) and Firsov et al. (1994). The former group demonstrated the PTX-sensitive component to be accountable for all of the reversal of inhibition observed, while the latter group found the PTX-sensitive component to account for only 40% of

the observed reversal of inhibition. Other mechanisms may therefore have a potential role in PGE_2-induced inhibition of AVP-dependent cAMP.

Results from our experiments showed virtually complete reversal of PGE_2-mediated inhibition of AVP-dependent cAMP using PTX, but because Firsov et al. (1994) was only able to achieve a 40% reversal with pertussis toxin, we still wanted to explore the possibility of EP_1 receptor subtype involvement and/or PKC activation. Noland et al. (1992) showed that the PGE_2-mediated inhibition of AVP-dependent cAMP in the cultured rabbit CCD had both PTX and staurosporine-sensitive components. This is suggestive of at least partial involvement of the EP_1 receptor subtype together with the EP_3 receptor subtype in modulating the inhibition observed in the CCD. Possibly a similar dual-inhibitory mechanism underlies PGE_2-mediated inhibition of AVP-dependent cAMP in the rat mTAL. It was pointed out earlier in the introduction that activated PKC (a potential product of EP_1 receptor subtype stimulation coupled to phosphatidyl inositol hydrolysis) may attenuate cAMP levels, demonstrating cell signaling cross-talk between different receptor subtypes. We decided to look at a PKC-dependent component to the inhibition using the PKC-specific inhibitors BIS.1 and calphostin C. Neither inhibitor was able to reduce PGE_2-dependent inhibition, suggesting that there is no cell signaling cross-talk occurring where PKC reverses PGE_2-mediated inhibition of AVP-dependent cAMP in our suspension of rat mTAL.

However, this still does not entirely rule out a role for the EP_1 receptor subtype. While there appears to be no PKC-dependent component, elevated intracellular Ca^{2+}, another consequence of PIP_2 hydrolysis, may be involved in reversing the inhibition of AVP-dependent cAMP. Hébert et al. 1991 showed the inhibition of sodium transport in the isolated

microperfused rabbit CCD to be partially dependent on increased levels of intracellular Ca^{2+} following addition of exogenous PGE_2. Firsov et al. (1994) had shown an increase in levels of intracellular calcium following incubation of rat mTAL with the PGE_2 precursor metabolite arachidonic acid. While we did not attempt to correlate changes in cAMP metabolism with measurable changes in intracellular Ca^{2+}, we did study the PGE_2-mediated inhibition of AVP-dependent cAMP in mTAL pretreated with the EP_1 receptor subtype specific antagonist AH-6809. There was no observable significant change in mTAL with and without pretreatment with AH-6809. Therefore the evidence provided to us by the experiments employing PTX, PKC-specific inhibitors and the EP_1 receptor subtype specific antagonist suggests that the inhibition of AVP-dependent cAMP by PGE_2 is coupled only through the Gi-regulatory protein as this inhibition is sensitive to PTX only.

We have further characterized the inhibitory E-prostanoid receptor subtype responsible for the inhibition of AVP-dependent cAMP observed as the EP_3 receptor subtype. So far our results have confirmed all previous work showing this inhibition in rat mTAL demonstrating that the mechanism of action to be through Gi-regulatory protein coupling. Results from our in situ hybridization experiments associate the expression of the EP_3 receptor subtype in the rat mTAL. Using ^{35}S-labelled oligoprobes, we were able to able to successfully associate positive labeling for the EP_3 receptor subtype mRNA with immunoperoxidase staining of TAL Tamm-Horsfall glycoprotein from serial sections of rat kidney. This correlates well with similar evidence showing expression of the EP_3 receptor subtype in mTAL from the mouse, human and rabbit kidneys (Breyer et al. 1993, Sugimoto et al. 1994, Breyer et al. 1996). The evidence from our in situ hybridization experiments is also in agreement with work done by Takeuchi et al. (1993) which

characterizes the EP_3 receptor subtype to the rat mTAL using RT-PCR. Careful analysis of the sections showing dark field illumination of EP_3 receptor subtype mRNA labeling at 100x magnification and the adjacent section showing bright field TH-immunoperoxidase staining of mTAL at 100x magnification demonstrates a clear similarity in tubule patterns. This evidence correlates well with our cAMP studies and therefore strongly suggests the presence of the EP_3 receptor subtype in the rat mTAL.

4.2 The EP_2 Receptor Subtype

We were able to produce a significant increase in cAMP using 10^{-7} and 10^{-6}M PGE_2 in our tissue preparation of rat mTAL. Elevations in cAMP responding to PGE_2 have been well characterized in rat mTAL (Torikai and Kurokawa 1983, Nakao et al. 1989). However, there are two E-prostanoid receptor subtypes which are Gs-regulatory protein coupled to adenylate cyclase (Coleman et al. 1984). The EP_2 receptor subtype stimulates smooth muscle relaxation and besides PGE_2, is also activated by the EP_2 receptor subtype specific agonist butaprost (Gardiner 1986, Coleman et al. 1994). While the EP_4 receptor subtype is also associated with smooth muscle relaxation, it is not activated by butaprost. The EP_4 receptor subtype was first found in piglet saphenous vein where it inhibits smooth muscle contraction (Coleman et al, 1994). At the protein level, there is 38% amino acid homology between EP_2 and EP_4 receptor subtypes (Breyer et al. 1996). The immediate questions facing us were which receptor subtype, EP_2 or EP_4, was responsible for the observed cAMP increase and was it possible to biochemically characterize this response. Although the cAMP-generating effect of PGE_2 in rat mTAL has been previously

demonstrated, no attempts have been made to characterize the receptor subtype involved in this response.

The EP_2 receptor subtype is involved in the PGE_2-dependent increase in cAMP. Butaprost caused a dose-dependent cAMP increase in our mTAL preparation. The EP_2 receptor subtype can be pharmacologically differentiated from EP_4 through its activation by butaprost. A significant increase in cAMP levels was observed at 10^{-6} and 10^{-5}M butaprost, the two highest concentrations tested. However, the physiological significance of the high cAMP levels attained with 10^{-5}M butaprost are suspect. Fig. 1 shows these cAMP levels to be 46% greater than the cAMP values attained using 10^{-5}M PGE_2, which were on a plateau with the cAMP levels measured at 10^{-6}M PGE_2. It is expected that at a given dose, the cAMP response to butaprost will be less than that observed for PGE_2 since the potency of butaprost for the EP_2 receptor subtype is ten fold weaker than that of PGE_2. The cAMP response curve to butaprost runs parallel to and less than the dose-response curve for PGE_2 at BUT concentrations less than 10^{-5}M. Both curves coincide well with the literature indicating the ten fold weaker potency of butaprost (Breyer et al. 1996). For example, the cAMP response to 10^{-7}M PGE_2 is not significantly different from that of 10^{-6}M butaprost. Using micromolar concentrations of butaprost, we were able to demonstrate an EP_2 receptor subtype-dependent component to the PGE_2-induced increase in cAMP.

There is no evidence implicating a role for the EP_4 receptor subtype in the PGE_2-dependent increase in cAMP. The fact that BUT increases cAMP suggests that the EP_2 receptor subtype is involved. This evidence however, still does not rule out a role for the EP_4 receptor subtype. Therefore experiments were designed to explore the involvement of the EP_4 receptor

subtype by attempting to reverse PGE_2 dependent cAMP with the EP_4 receptor subtype antagonist AH-23848B at a concentration of 10μM. No inhibition of the PGE_2-dependent increase in cAMP was observed using AH-23848B. While this suggests that the EP_4 receptor subtype is not involved, it is important to keep in mind that AH-23848B is a weak EP_4 receptor subtype antagonist. This, combined with the cAMP response curve to butaprost, leads us to believe that the EP_2 receptor subtype alone is responsible for the PGE_2-dependent increases in cAMP observed in our mTAL preparation.

Although our cell signaling evidence characterizes the EP_2 receptor subtype in the mTAL, results from in situ hybridization experiments looking for expression of this receptor subtype do not support this. Due to 5-10% impurity of our tissue preparation any receptor or receptor subtype for which cell signaling evidence is observed would be further characterized using in situ hybridization to look for the expression of that receptor or receptor subtype. Nowhere in the outer medullary regions of our rat kidney sections was there observed positive labeling for the EP_2 receptor subtype mRNA. Although perplexing, the fact that our in situ data does not corroborate our cAMP data is consistent with recent literature reviews specifically addressing this topic. Two recent literature reviews outline evidence showing PGE_2-dependent cAMP production in rat mTAL while localization of mRNA for EP_2 and EP_4 receptor subtypes occurs in glomeruli only in mouse and human kidneys respectively (Breyer et al. 1995, Breyer et al. 1996).

The PGE_2-dependent cAMP responses observed may be occurring at non-mTAL segments in the outer medulla. Outer medullary tubule segments other than the mTAL have been shown to produce cAMP in response to PGE_2. PGE_2-dependent cAMP production has been shown in both the rat thin descending limb of Henle and in the medullary collecting duct (Torikai

and Kurokawa 1984, Torikai and Kurokawa 1981, Sonnenberg and Smith 1988, Hébert et al. 1988, Nakao et al. 1989). It may be that the cAMP responses observed in our mTAL tissue preparation are indeed coming from tubule segments other than the mTAL. Contamination by other tubule segments in the mTAL suspension may include portions of the thin limb and collecting duct. Assuming the PGE_2-dependent cAMP responses observed represent events occurring at non-mTAL segments in the outer medulla, in situ hybridization should still detect expression of the EP_2 receptor subtype in the outer medulla. Two reasons may explain why there is no visible detection of positive labeling for the EP_2 receptor subtype mRNA under dark field or bright field microscopy. First, expression of EP_2 receptor subtype mRNA may occur at very low levels. Previous work highlights the lower level of renal expression of the EP_2 receptor subtype compared to the high levels of expression for the EP_3 receptor subtype (Breyer et al 1993, Takeuchi et al 1993). Secondly, the diameter and number of various nephron segments crossing the outer medulla may play a role. The mTAL may dominate the visual field under light microscopy; these structures may reduce detection of EP_2 receptor subtype mRNA in the thinner outer medullary collecting duct and thin descending limb. All of the inner cortical nephrons have TAL, but only 1/8 of these nephrons have thin descending limbs. Therefore, the sensitivity of the in situ hybridization procedure may not be high enough to detect low levels of EP_2 receptor subtype expression and/or a weak labeling may be masked by the dominant mTAL segments in the outer medulla. Lastly, in the absence of labelling of the EP_2 oligoprobe in a control tissue which expresses this receptor, it is possible this probe does not bind its intended target.

4.3 The IP Receptor

CyclicAMP generating response to PGI_2 have been characterized in cultured rabbit IMCD cells (Veis et al. 1990). Although not stated in the study, this cAMP response is indicative of a putative IP_2 receptor subtype mediated event. Hébert et al. (1995) was the first to find evidence suggesting the presence of putative IP_1 and IP_3 receptor subtypes in the rabbit CCD. Using carba prostacyclin (c-PGI_2) and ILP, he was able to demonstrate liberation of intracellular Ca^{2+} and inhibition of AVP-dependent hydraulic conductivity in rabbit CCD. However, no work has been done to characterize second messenger responses to PGI_2 analogues in the mTAL.

Both PGE_2 and PGI_2 stimulate an increase in cAMP in the collecting duct. In this region of the nephron, the second messenger responses to PGE_2 and PGI_2 may compliment each other. Therefore, because PGE_2 stimulates an elevation in cAMP in the mTAL, PGI_2 analogs may augment this response as well. However, there were no cAMP generating responses observed in freshly isolated suspension of rat mTAL using the PGI_2 analogs ILP and CCP. Even at 10^{-5}M ILP and CCP, concentrations approaching pharmacological levels, no change was observed in intracellular cAMP content compared to control values.

Iloprost and CCP caused an inhibition of AVP-dependent cAMP production in our freshly isolated suspension of rat mTAL. These experiments were performed in identical fashion to those studying the similar inhibition by PGE_2 with the exception of the prostanoid used. Both ILP and PGE_2 inhibit AVP-dependent hydraulic conductivity in the collecting duct. In the case of PGE_2 this is mediated in part through a Gi-regulatory protein attenuating AVP-dependent cAMP.

Through a similar mechanism of action, PGE_2 inhibits AVP-dependent NaCl reabsorption across the mTAL. The rationale for testing for a similar response to PGI_2 was perhaps this prostanoid performs a role complimentary to PGE_2 in this region of the nephron. Some differences between the two PGI_2 analogues with respect to potency and percent inhibition of AVP-dependent cAMP we observed. A maximal inhibition of 80% was achieved using ILP at a concentration of 10^{-7}M. For CCP a maximal inhibition of 68.9% was obtained but at a concentration of 10^{-6}M. The mTAL suspension was pretreated with the PDE inhibitor Ro20-1724, suggesting the PGI_2-analogue dependent inhibition to be a result of attenuated synthesis of cAMP.

The possibility that the inhibitory action of two PGI_2 analogs was actually mediated through the EP_3 receptor subtype had to be considered. To distinguish the PGE_2 and PGI_2 analog-dependent inhibitors as being mediated through separate and distinct receptors, additivity experiments were performed. Similar experiments were done by Hébert to distinguish a putative IP receptor from the corresponding EP receptor subtype in studies conducted in the rabbit CCD. Tubules were pretreated with ILP prior to incubation with PGE_2 because observed that PGE_2 could bind but not activate the IP receptor. Therefore, we used the same sequence of addition of prostanoids was used in the present study. These experiments were designed to test for additivity of inhibition of AVP-dependent cAMP when PGE_2 and ILP were incubated together compared to either incubated alone. No additivity of inhibition was observed. However, although this result does not distinguish the inhibitory responses as mediated through separate receptors, it does not mean the ILP-dependent inhibition is mediated through non-specific activity at the EP_3 receptor subtype.

The mechanism of action underlying the PGE_2 analogue-mediated inhibition of AVP-dependent cAMP is coupled through the Gi regulatory protein. Pretreatment with 1 ug/ml PTX for 60 minutes at 37°C resulted in 72 and 73% reversal of inhibition of AVP-dependent cAMP by ILP and CCP respectively. These experiments had the potential to distinguish the IP receptor from the EP_3 receptor subtype-dependent inhibition if the mechanism of action was different. However, evidence from these experiments suggests that the inhibitory response is, like the PGE_2-mediated inhibition, coupled through a Gi-regulatory protein. Therefore, results from the mechanism of action experiments do not facilitate the differentiation of the ILP and CCP-mediated inhibition of AVP-dependent cAMP as occurring through a receptor separate and distinct from the EP_3 receptor subtype-mediated response. The levels of cAMP achieved by reversal of inhibition with PTX are not significantly different from those when the mTAL were incubated with AVP alone. Therefore, this suggests that the reversal of inhibition obtained using PTX is complete.

Also, there appears to be no PKC-dependent component involved in the ILP and CCP-mediated inhibition of AVP-dependent cAMP. The complete reversal of this inhibition by PTX suggests that Gi-regulatory protein coupling is the only underlying mechanism. However, a PKC-dependent component underlying the inhibition of AVP-dependent cAMP was examined. Using the PKC-specific inhibitors Bis.1 for ILP and CCP-dependent inhibition and calphostin C for ILP-dependent inhibition, PKC-dependent inhibition of AVP-dependent cAMP through cell signaling cross-talk was tested. No reversal of inhibition of AVP-dependent cAMP was observed. Although we were not looking at possible IP-dependent effects by measuring changes in PKC directly, we were studying these effects indirectly by looking at cell signaling cross-talk in the

form of PKC-dependent inhibition of cAMP. Based on this evidence and that from the experiments using PTX, we propose that the mechanism of action underlying the PGI_2 analog-mediated inhibition of AVP-dependent cAMP is coupled mainly through the Gi-regulatory protein.

Determining relative potency from the concentration of prostanoid where maximal cAMP response is achieved, ILP appears to have a greater potency than CCP. Comparison of the dose-response inhibition curves for the two analogs shows that maximal inhibition was achieved at 10^{-7}M ILP and 10^{-6}M CCP. Cicaprost is reported to be highly specific for the IP receptor and has no affinity for other prostanoid receptor and receptor subtypes tested. Iloprost on the other hand, is less specific as it has been shown to exhibit agonist activity for the EP_1 receptor subtype. Sturzebecher et al. 1985 has shown that CCP is at least as potent as iloprost with regards to changes in cAMP metabolism, vasodilating, anti-thrombotic and platelet-activation inhibiting effects in vivo in human and rat. However, CCP has greater "net"-potency due to a longer biological activity resulting from enhanced chemical and metabolic stability. The CCP molecule is not as susceptible to β-oxidation as is iloprost. In our experiments, we observe a reverse order of potency for ILP and CCP compared to that reported in the literature.

One reason the potency of ILP may appear greater than that of CCP may is that ILP may stimulate an EP_1 receptor subtype on the OMCD leading to a second messenger response such as elevated PKC activity, which attenuates AVP-dependent cAMP through cell signaling cross-talk. This may be a possibility if there is 5-10% contamination from non-mTAL segments in the mTAL suspension, and if a significant portion of this of this contamination is OMCD. However, noticed from our experiments using the EP_1 receptor subtype specific antagonist AH-6809 showed was

no EP_1-dependent effect in either the PGE_2 or ILP-mediated inhibition of AVP-dependent cAMP in the rat mTAL suspension. The EP_1 receptor subtype has been characterized to the OMCD (Breyer et al, 1996); therefore, with sufficient collecting duct contamination one might have observed an EP_1-dependent effect in response to both ILP and PGE_2. The apparent greater potency of ILP over CCP probably does not come from a potential 5-10% contamination from the collecting duct.

It is unclear why a reverse order of potency to that reported for ILP and CCP was observed. It may be that the potency observed is characteristic of rat renal tissue and therefore not representative of what is taking place in cells of the blood-vascular system, where the above observations from previous studies were made. Studies showing expression of IP receptor cDNA in both Chinese hamster ovary (CHO) and COS-7 cells show CCP to have a potency equal to or greater than that of ILP (Namba et al. 1993, Katsuyama et al. 1994, Sasaki et al. 1994). Further studies which look at the agonist potency of the IP receptor in renal cell culture will need to be done to address this issue.

Intrarenal localization of prostanoid receptor expression has been done for the E-prostanoid receptor subtypes in mouse, human and rabbit kidney. Expression for the EP_1 receptor subtype has been shown in the rabbit and mouse collecting duct (Sugimoto et al. 1994, Fowler et al. 1995). Sugimoto et al. (1994) has also demonstrated EP_2 receptor subtype expression in the mouse glomerulus, while Breyer et al. (1996) has demonstrated expression of the EP_4 receptor subtype in the rabbit glomerulus. EP_3 receptor subtype expression has been demonstrated in the TAL and the collecting duct of various species (Sugimoto et al. 1994, Breyer et al. 1993, Breyer

et al. 1996). Our studies demonstrate similar localization of EP_3 receptor subtype expression in the rat mTAL.

Very little work has been done studying renal expression of the I-prostanoid receptor The receptor has been cloned from human, mouse and rat (Namba et al. 1993, Katsuyama et al. 1994, Sasaki et al. 1994). Northern blot analysis from these studies has demonstrated expression of the IP receptor in the lung, spleen, heart, pancreas, thymus, stomach, and aorta. These were the first studies to clone the IP receptor; however, in their search for tissue expression, they did not discuss the kidney.

In situ hybridization using ^{35}S-labelled riboprobes on frozen sections of mouse tissue sections has been done previously. In this work, Oida et al. 1995 has shown the expression of the IP receptor subtype in the thymus, spleen, heart, lung, central nervous system and kidney. In the kidney, they show IP receptor expression in the smooth muscle cells of the renal and interlobular arteries and in the smooth muscle cells of the afferent arteriole. While their results demonstrate expression for the IP receptor in the renal vascular, they do not demonstrate this in any tubular structures of the nephron, where second messenger evidence suggests the presence of this receptor. Species differences and/or differences in in situ methodology may account for the lack of IP receptor expression in nephron segments observed by this group.

Our results demonstrate for the first time the presence of an IP receptor in the rat mTAL coupled to the Gi-regulatory protein. Careful comparison of the 100x dark field photomicrograph of IP receptor positive labeling shows identical tubule pattern with that in the 100x bright field photomicrograph of TH-immunoperoxidase staining in the outer medulla. While specific positive labeling of the IP receptor radiooligoprobe was observed on the rat mTAL, specific labeling

above background in any other regions of the rat kidney was not observed. The correlation the cAMP results and in situ hybridization experiments provide evidence for the existence of an IP receptor coupled to Gs in the rat mTAL.

The inhibitory responses of AVP-dependent cAMP due to the PGI_2 analogs tested are mediated through an IP receptor which is separate and distinct from the EP_3 receptor subtype. The experiments discussed up to this point were based on the study of cAMP second messenger responses. The experiments designed to look at potential additivity of inhibition between PGE_2 and ILP-mediated inhibition of AVP-dependent cAMP were not successful in differentiating the IP receptor mediated inhibitory response from that of the EP_3 receptor subtype. Since both the IP receptor and EP_3 receptor subtype-mediated response couple through the PTX-sensitive Gi-regulatory protein, we were unable to distinguish the two based on mechanism of action. However, our final set of experiments involving association of receptor expression to the mTAL was successful in demonstrating the differential expression of the IP receptor and EP_3 receptor subtype.

This is the first study to characterize the IP receptor in the mTAL of the rat kidney. The approach involved two methods: measuring changes in cAMP metabolism induced by the PGI_2 analogs ILP and CCP by radioimmunoassay and demonstrating IP receptor expression by in situ hybridization. While significant inhibition of AVP-dependent cAMP was achieved with ILP and CCP, it was unclear if this response was due to an EP_3 receptor subtype mediated event or if the OMCD was involved due to potential 5-10% contamination in the isolated suspension of mTAL. Therefore, further characterization of the PGI_2 analog-dependent response was done using in situ hybridization.

In conclusion, changes in cAMP were measured following incubation of a freshly isolated suspension of rat mTAL with various prostanoid agonists and antagonists. Using this mTAL suspension responses of the of the EP_3 receptor subtype and the IP receptor were characterized. While this has been shown in the case of the EP_3 receptor subtype, these experiments were necessary to validate the biological model to conduct similar studies of the IP receptor. This is the first study demonstrating a PTX-sensitive PGI_2 analogue-dependent inhibition of cAMP and expression for the IP receptor in a tubule section of the nephron, and more specifically, the mTAL.

Section 5: References

5.0 References

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